

27

Hemoglobinopathies (Structural Defects in Hemoglobin)

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OUTLINE

Structure of Globin Genes Hemoglobin Development Genetic Mutations

Zygoty Pathophysiology Nomenclature

Hemoglobin S

*Sickle Cell Anemia
Sickle Cell Trait*

Hemoglobin C

*Prevalence, Etiology, and
Pathophysiology*

Hemoglobin C-Harlem (Hemoglobin C-George- town)

Hemoglobin E

*Prevalence, Etiology, and
Pathophysiology
Clinical Features*

Hemoglobin O-Arab

Hemoglobin D and Hemo- globin G

Compound Heterozygosity with Hemoglobin S and Another β -Globin Gene Mutation

*Hemoglobin SC
Hemoglobin S β -Thalasse-
mia –
Hemoglobin SD and Hemo-
globin SG-Philadelphia
Hemoglobin S/O-Arab and
HbS/D-Punjab
Hemoglobin S-Korle Bu*

Concomitant Cis Muta- tions with Hemoglobin S

*Hemoglobin C-Harlem
Hemoglobin S-Antilles and
Hemoglobin S-Oman*

Hemoglobin M

Unstable Hemoglobin Variants

Clinical Features

OBJECTIVES

After completion of this chapter, the reader will be able to:

1. Explain the difference between structural hemoglobin disorders and thalassemias, and describe the types of mutations found in the structural disorders.
2. Describe globin gene structure and the development of normal human hemoglobins throughout prenatal and postnatal life.
3. Differentiate between homozygous and heterozygous states and the terms “disease” and “trait” as they relate to the hemoglobinopathies.
4. Given the hemoglobin genotypes of parents involving common β chain variants, determine the possible genotypes of their children using a Punnett square.
5. Describe the general geographic distribution of common hemoglobin variants and the relationship of that distribution with the prevalence of malaria and glucose-6-phosphate dehydrogenase deficiency.
6. For disorders involving Hb S and Hb C, describe the genetic mutation, the effect of the mutation on the hemoglobin molecule, the inheritance pattern, pathophysiology, symptoms, clinical findings, peripheral blood findings, laboratory diagnosis, and genetic counseling and treatment considerations.
7. Describe the genetic mutation, clinical findings, and laboratory diagnosis for disorders involving Hb C-Harlem, Hb E, Hb O-Arab, Hb D, and Hb G.
8. Describe the clinical and laboratory findings for the compound heterozygous disorders of Hb S with Hb C, β -thalassemia, Hb D, Hb O-Arab, Hb Korle Bu, and Hb C-Harlem.
9. Describe the electrophoretic mobility of Hb A, Hb F, Hb S, and Hb C at an alkaline pH, and explain how other methods (including the Hb S solubility test, citrate agar electrophoresis at acid pH, and high-performance liquid chromatography) are used to distinguish Hb S and Hb C from other hemoglobins with the same mobility.
10. Describe the genetic mutations, inheritance patterns, pathophysiology, and clinical and laboratory findings in hemoglobin variants that result in methemoglobinemia.
11. Describe the inheritance patterns, causes, and clinical and laboratory findings of unstable hemoglobin variants.
12. Discuss the pathophysiology of hemoglobin variants with increased and decreased oxygen affinities, and explain how they differ from unstable hemoglobins.
13. Given a case history and clinical and laboratory findings, interpret test results to identify the hemoglobin variants present in the patient.

CASE STUDY

After studying the material in this chapter, the reader should be able to respond to the following case study:

An 18-year-old African-American woman was seen in the emergency department for fever and abdominal pain. The following results were obtained on a complete blood count:

	Patient Results	Reference Interval	Patient Results	Reference Interval
HCT (%)	32.5	35–49		
RDW (%)	19.5	11.5–14.5		
Platelets ($\times 10^9/L$)	410	150–450		
Segmented neutrophils (%)	75	50–70		
WBCs ($\times 10^9/L$)	11.9	3.6–10.6		
RBCs ($\times 10^{12}/L$)	3.67	4.00–5.40		
HGB (g/dL)	10.9	12.0–15.0		
Lymphocytes (%)	18	18–42		
Monocytes (%)	3	2–11		

OUTLINE—cont'd

Treatment and Prognosis
**Hemoglobins with In-
 creased and Decreased
 Oxygen Affinity**
 Hemoglobins with Increased
 Oxygen Affinity
 Hemoglobins with De-
 creased Oxygen Affinity
Global Burden of Hemo-

CASE STUDY—cont'd

After studying the material in this chapter, the reader should be able to respond to the following case study:

	Patient Results	Reference Interval
Eosinophils (%)	3	1–3
Basophils (%)	1	0–2
Reticulocytes (%)	3.1	0.5–2.5

A typical field in the patient's peripheral blood film is shown in Figure 27-1. Electrophoresis on cellulose acetate at alkaline pH showed 50.9% Hb S and 49.1% Hb C.

1. Select confirmatory tests that should be performed and describe the expected results.
2. Describe the characteristic red blood cell morphology on the peripheral blood film.
3. Based on the electrophoresis and red blood cell morphology results, what diagnosis is suggested?
4. If this patient were to marry a person of genotype Hb AS, what would be the expected frequency of genotypes for each of four children?

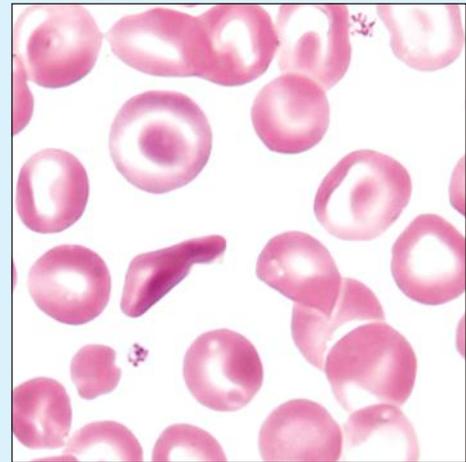


Figure 27-1 Peripheral blood film for the patient in the case study ($\times 1000$). (Courtesy Ann Bell, University of Tennessee, Memphis.)

Hemoglobinopathy refers to a disease state (*opathy*) involving the hemoglobin (Hb) molecule. Hemoglobinopathies are the most common genetic diseases, affecting approximately 7% of the world's population.¹ Approximately 300,000 children are born each year with some form of inherited hemoglobin disorder.² All hemoglobinopathies result from a genetic mutation in one or more genes that affect hemoglobin synthesis. The genes that are mutated can code for either the proteins that make up the hemoglobin molecule (globin or polypeptide chains) or the proteins involved in synthesizing or regulating synthesis of the globin chains. Regardless of the mutation encountered, all hemoglobinopathies affect hemoglobin synthesis in one of two ways: qualitatively or quantitatively. In qualitative hemoglobinopathies, hemoglobin synthesis occurs at a normal or near-normal rate, but the hemoglobin molecule has an altered amino acid sequence within the globin chains. This change in amino acid sequence alters the structure of the hemoglobin molecule (structural defect) and its function (qualitative defect). In contrast, thalassemias result in a reduced rate of hemoglobin synthesis (quantitative) but do not affect the amino acid sequence of the globin chains. A reduction in the amount of hemoglobin synthesized produces an anemia and stimulates the production of other hemoglobins not affected by the mutation in an attempt to compensate for the anemia. Based on this distinction, hematologists divide hemoglobinopathies into two categories: structural defects (qualitative) and thalassemias (quantitative). To add confusion to the classification scheme, many hematologists also refer to *only* the structural defects as hemoglobinopathies. This chapter describes the structural or qualitative defects that are referred to as *hemoglobinopathies*; the quantitative defects (thalassemias) are described in Chapter 28.

STRUCTURE OF GLOBIN GENES

As discussed in Chapter 10, there are six functional human globin genes located on two different chromosomes. Two of the globin genes, α and ζ , are located on chromosome 16 and are referred to as α -like genes. The remaining four globin genes, β , γ , δ , and ϵ , are located on chromosome 11 and are referred to as β -like genes. In the human genome, there is one copy of each globin gene per chromatid, for a total of two genes per diploid nucleus, with the exception of α and γ . There are two copies of the α and γ genes per chromatid, for a total of four genes per diploid nucleus. Each globin gene codes for the corresponding globin chain: the α -globin genes (*HBA1* and *HBA2*) are used as the template to synthesize the α -globin chains, the β -globin gene (*HBB*) codes for the β -globin chain, the γ -globin genes (*HBG1* and *HBG2*) code for the γ -globin chains, and the δ -globin gene (*HBD*) codes for the δ -globin chain.

HEMOGLOBIN DEVELOPMENT

Each human hemoglobin molecule is composed of four globin chains: a pair of α -like chains and a pair of β -like chains. During the first 3 months of embryonic life, only one α -like gene (ζ) and one β -like gene (ϵ) are activated, which results in the production of ζ and ϵ globin chains that pair to form hemoglobin Gower-1 ($\zeta_2\epsilon_2$). Shortly thereafter, α and γ chain synthesis begins, which leads to the production of Hb Gower-2 ($\alpha_2\epsilon_2$) and Hb Portland ($\zeta_2\gamma_2$). Later in fetal development, ζ and ϵ synthesis ceases; this leaves α and γ chains, which pair to produce Hb F ($\alpha_2\gamma_2$), also known as *fetal hemoglobin*. During the 6 months after birth, γ chain synthesis gradually decreases and is replaced by β chain synthesis so that Hb A ($\alpha_2\beta_2$), also

known as *adult hemoglobin*, is produced. Recent evidence suggests BCL11A and KLF1, zinc-finger transcriptional repressors, are necessary to silence the γ -globin gene and mutations in the gene that codes for either factor results in elevated HbF levels.³ The remaining globin gene, δ , becomes activated around birth, producing δ chains at low levels that pair with α chains to produce the second adult hemoglobin, Hb A₂ ($\alpha_2\delta_2$). Normal adults produce Hb A (95%), Hb A₂ (less than 3.5%), and Hb F (less than 1% to 2%).

GENETIC MUTATIONS

More than 1000 structural hemoglobin variants (hemoglobinopathies) are known to exist throughout the world, and more are being discovered regularly (Table 27-1).^{4,5} Each of these hemoglobin variants results from one or more genetic mutations that alter the amino acid sequence. Some of these changes alter the molecular structure of the hemoglobin molecule, ultimately affecting hemoglobin function. The types of genetic mutations that occur in the hemoglobinopathies include point mutations, deletions, insertions, and fusions involving one or more of the adult globin genes— α , β , γ , and δ .⁵

Point mutation is the most common type of genetic mutation occurring in the hemoglobinopathies. Point mutation is the replacement of one original nucleotide in the normal gene with a different nucleotide. Because one nucleotide is replaced by one nucleotide, the codon triplet remains intact, and the reading frame is unaltered. This results in the substitution of one amino acid in the globin chain product at the position corresponding to the location of the original point mutation. As can be seen in Table 27-1, 1109 of the 1181 known hemoglobin variants result from a point mutation that causes an amino acid substitution. It also is possible to have two point mutations occurring in the same globin gene, which results in two amino acid substitutions within the same globin chain. Over 35 mutations occur by this mechanism.⁵

TABLE 27-1 Molecular Abnormalities of Hemoglobin Variants

	NUMBER OF VARIANTS BY GLOBIN CHAIN				
	α	β	δ	γ	Total
Amino acid substitution	413	535	65	96	1109
Deletions or insertions	22	48	1	1	72
Total	435	583	66	97	1181
Fusions	—	—	—	—	9*

*Seven fusions involve the β and δ chains; two fusions involve the β and γ chains. Data from Patrinos GP, Giardine B, Riemer W, et al: Improvements in the HbVar database of human hemoglobin variants and thalassemia mutations for population and sequence variation studies, *Nucl Acids Res* 32(database issue):D537-541, 2004. Available at: <http://globin.cse.psu.edu/hbvar/menu.html>. Accessed November 29, 2013.

The table is designed to provide a relative distribution of mutation types and just includes structural variants. Fifty-one of the variants are also categorized as thalassemias. Mutations are being added regularly.

Deletions involve the removal of one or more nucleotides, whereas insertions result in the addition of one or more nucleotides. Usually deletions and insertions are not divisible by three and disrupt the reading frame, which leads to the nullification of synthesis of the corresponding globin chain. This is the case for the quantitative thalassemias (Chapter 28). In hemoglobinopathies, the reading frame usually remains intact, however; the result is the addition or deletion of one or more amino acids in the globin chain product, which sometimes affects the structure and function of the hemoglobin molecule. Of the 1181 variants described in Table 27-1, 72 variants result from deletions or insertions, or both.⁵

Chain extensions occur when the stop codon is mutated so that translation continues beyond the typical last codon. Amino acids continue to be added until a stop codon is reached by chance. This process produces globin chains that are longer than normal. Significant globin chain extensions usually result in degradation of the globin chain and a quantitative defect. If the extension of the globin chain is insufficient to produce significant degradation, however, the defect is qualitative and is classified as a hemoglobinopathy. Hemoglobin molecules with extended globin chains fold inappropriately, which affects hemoglobin structure and function.

Gene fusions occur when two normal genes break between nucleotides, switch positions, and anneal to the opposite gene. For example, if a β -globin gene and a δ -globin gene break in similar locations, switch positions, and reanneal, the resultant genes would be $\beta\delta$ and $\delta\beta$ fusion genes in which the head of the fusion gene is from one original gene and the tail is from the other. As long as the reading frames are not disrupted and the globin chain lengths are similar, the genes are transcribed and translated into hybrid globin chains. The fusion chains fold differently, however, and affect the corresponding hemoglobin function. Nine fusion globin chains have been identified (see Table 27-1).

ZYGOSITY

Zygoty refers to the association between the number of gene mutations and the level of severity of the resultant genetic defect. Generally, there is a level of severity associated with each gene that is normally used to synthesize the globin chain product. For the normal adult globin genes, there are four copies of the α and γ genes and two copies of the β and δ genes. In theory, this could result in four levels of severity for α and γ gene mutations and two levels of severity for the β and δ gene mutations. Expressed another way, if all things were equal, it would require twice as many mutations within the α and γ genes to produce the same physiologic effect as mutations within the β and δ genes. Because the γ and δ genes are transcribed and translated at such low levels in adults, however, mutations of either gene would have little impact on overall hemoglobin function. In addition, because the dominant hemoglobin in adults, Hb A, is composed of α and β chains, β gene mutations would affect overall hemoglobin function to a greater extent than the same number of α gene mutations. This partially explains the greater number of identified β chain

variants compared with α chain variants, because a single β gene mutation would be more likely to create a clinical condition than would a single α gene mutation.

The inheritance pattern of β chain variants is referred to as *heterozygous* when only one β gene is mutated and *homozygous* when both β genes are mutated. The terms *disease* and *trait* are also commonly used to refer to the homozygous (disease) and heterozygous (trait) states.

PATHOPHYSIOLOGY

Pathophysiology refers to the manner in which a disorder translates into clinical symptoms. The impact of point mutations on hemoglobin function depends on the chemical nature of the substituted amino acid, where it is located in the globin chain, and the number of genes mutated (*zygosity*). The charge and size of the substituted amino acid may alter the manner in which the globin chain folds. A change in charge affects the interaction of the substituted amino acid with adjacent amino acids. In addition, the size of the substituted amino acid makes the globin chain either more or less bulky. Therefore, the charge and the size of the substituted amino acid determine its impact on hemoglobin structure by potentially altering the tertiary structure of the globin chain and the quaternary structure of the hemoglobin molecule. Changes in hemoglobin structure usually affect function. Location of the substitution within the globin chain also has an impact on the degree of structural alteration and hemoglobin function based on its positioning within the molecule and the interactions with the surrounding amino acids. In the case of the sickle cell mutation, one amino acid substitution results in hemoglobin polymerization, leading to the formation of long hemoglobin crystals that stretch the red blood cell (RBC) membrane and produce the characteristic crescent moon or sickle cell shape.

Zygoty also affects the pathophysiology of the disease. In β -hemoglobinopathies, *zygoty* predicts two severities of disease. In homozygous β -hemoglobinopathies, in which both β genes are mutated, the variant hemoglobin becomes the dominant hemoglobin type, and normal hemoglobin (Hb A) is absent. Examples are sickle cell disease (SCD, Hb SS) and Hb C disease (Hb CC). In heterozygous β -hemoglobinopathies, one β gene is mutated and the other is normal, which suggests a 50/50 distribution. In an attempt to minimize the impact of the abnormal hemoglobin, however, the variant hemoglobin is usually present in lesser amounts than Hb A. Nevertheless, in some cases they may be present in equal amounts. Examples are Hb S trait (Hb AS) and Hb C trait (Hb AC). Patients with homozygous sickle cell disease (Hb SS) inherit a severe form of the disease that occurs less frequently but requires lifelong medical intervention, which must begin early in life, whereas heterozygotes (Hb AS) are much more common but rarely experience symptoms.

Fishleder and Hoffman⁶ divided the structural hemoglobins into four groups: abnormal hemoglobins that result in hemolytic anemia, such as Hb S and the unstable hemoglobins; abnormal hemoglobins that result in methemoglobinemia, such as Hb M; hemoglobins with either increased or decreased

oxygen affinity; and abnormal hemoglobins with no clinical or functional effect. Imbalanced chain production also may be associated in rare instances with a structurally abnormal chain, such as Hb Lepore,⁴ because of the reduced production of the abnormal chain. The functional classification of selected hemoglobin variants is summarized in Box 27-1.

Many of the variants are clinically insignificant because they do not show any physiologic effect. As discussed previously, most clinical abnormalities are associated with the β chain followed by the α chain. Involvement of the γ and δ chains does occur, but because of the small amount of hemoglobin involved, it is rarely detected and is usually of no consequence. Box 27-2 lists clinically significant abnormal hemoglobins. The most frequently occurring of the abnormal hemoglobins and the most severe is Hb S.

NOMENCLATURE

As hemoglobins were reported in the literature, they were designated by letters of the alphabet. Normal adult hemoglobin and fetal hemoglobin were called *Hb A* and *Hb F*. By the time the middle of the alphabet was reached, however, it became apparent that the alphabet would be exhausted before all mutations were named. Currently, some abnormal hemoglobins are assigned a common designation and a scientific designation. The common name is selected by the discoverer and usually represents the geographic area where the hemoglobin was identified. A single capital letter is used to indicate a special characteristic of the hemoglobin variants, such as hemoglobins demonstrating identical electrophoretic mobility but containing different amino acid substitutions, as in Hb G-Philadelphia, Hb G-Copenhagen, and Hb C-Harlem. The variant description also can involve scientific designations that indicate the variant chain, the sequential and the helical number of the abnormal amino acid, and the nature of the substitution. The designation [β_6 (A_3) Glu→Val] for the Hb S mutation indicates the substitution of valine for glutamic acid in the A helix in the β chain at position 6.⁴

HEMOGLOBIN S

Sickle Cell Anemia

History

Although the origin of sickle cell anemia has not been identified, symptoms of the disease have been traced in one Ghanaian family back to 1670.⁷ Sickle cell anemia was first reported by a Chicago cardiologist, Herrick, in 1910 in a West Indian student with severe anemia. In 1917, Emmel recorded that sickling occurred in nonanemic patients and in patients who were severely anemic. In 1927, Hahn and Gillespie described the pathologic basis of the disorder and its relationship to the hemoglobin molecule. These investigators showed that sickling occurred when a solution of RBCs was deficient in oxygen and that the shape of the RBCs was reversible when that solution was oxygenated again.^{4,8} In 1946, Beet reported that malarial parasites were present less frequently in blood films from patients with SCD than in individuals without SCD.⁹ It was determined that the sickle cell trait confers a resistance against infection with *Plasmodium falciparum*

BOX 27-1 Functional Classification of Selected Hemoglobin (Hb) Variants**I. Homozygous: Hemoglobin Polymorphisms: The Variants That Are Most Common**

Hb S: $\alpha_2\beta_2^{6\text{Val}}$ —severe hemolytic anemia; sickling

Hb C: $\alpha_2\beta_2^{6\text{Lys}}$ —mild hemolytic anemia

Hb D-Punjab: $\alpha_2\beta_2^{121\text{Gln}}$ —no anemia

Hb E: $\alpha_2\beta_2^{26\text{Lys}}$ —mild microcytic anemia

II. Heterozygous: Hemoglobin Variants Causing Functional Aberrations or Hemolytic Anemia in the Heterozygous State**A. Hemoglobins Associated with Methemoglobinemia and Cyanosis**

1. Hb M-Boston: $\alpha_2\beta_2^{58\text{Tyr}}$

2. Hb M-Iwate: $\alpha_2\beta_2^{87\text{Tyr}}$

3. Hb Auckland: $\alpha_2\beta_2^{87\text{Asn}}$

4. Hb Chile: $\alpha_2\beta_2^{28\text{Met}}$

5. Hb M-Saskatoon: $\alpha_2\beta_2^{63\text{Tyr}}$

6. Hb M-Milwaukee-1: $\alpha_2\beta_2^{67\text{Glu}}$

7. Hb M-Milwaukee-2: $\alpha_2\beta_2^{92\text{Tyr}}$

8. Hb F-M-Osaka: $\alpha_2\gamma_2^{63\text{Tyr}}$

9. Hb F-M-Fort Ripley: $\alpha_2\gamma_2^{92\text{Tyr}}$

B. Hemoglobins Associated with Altered Oxygen Affinity

1. Increased affinity and erythrocytosis

a. Hb Chesapeake: $\alpha_2\beta_2^{92\text{Leu}}$

b. Hb J-Capetown: $\alpha_2\beta_2^{92\text{Gln}}$

c. Hb Malmo: $\alpha_2\beta_2^{97\text{Gln}}$

d. Hb Yakima: $\alpha_2\beta_2^{99\text{His}}$

e. Hb Kempsey: $\alpha_2\beta_2^{99\text{Asn}}$

f. Hb Ypsi (Ypsilanti): $\alpha_2\beta_2^{99\text{Tyr}}$

g. Hb Hiroshima: $\alpha_2\beta_2^{146\text{Asp}}$

h. Hb Rainier: $\alpha_2\beta_2^{145\text{Cys}}$

i. Hb Bethesda: $\alpha_2\beta_2^{145\text{His}}$

2. Decreased affinity—may have mild anemia or cyanosis

a. Hb Kansas: $\alpha_2\beta_2^{102\text{Thr}}$

b. Hb Titusville: $\alpha_2\beta_2^{94\text{Asn}}$

c. Hb Providence: $\alpha_2\beta_2^{82\text{Asn}}$

d. Hb Agenogi: $\alpha_2\beta_2^{90\text{Lys}}$

e. Hb Beth Israel: $\alpha_2\beta_2^{102\text{Ser}}$

f. Hb Yoshizuka: $\alpha_2\beta_2^{108\text{Asp}}$

C. Unstable Hemoglobins

1. Hemoglobin may precipitate as Heinz bodies after splenectomy (congenital Heinz body anemia)

a. Severe hemolysis: no improvement after splenectomy

Hb Bibba: $\alpha_2\beta_2^{136\text{Pro}}$

Hb Hammersmith: $\alpha_2\beta_2^{42\text{Ser}}$

Hb Bristol-Alesha: $\alpha_2\beta_2^{67\text{Asp}}$ or 67Met

Hb Olmsted: $\alpha_2\beta_2^{141\text{Arg}}$

b. Severe hemolysis: improvement after splenectomy

Hb Torino: $\alpha_2\beta_2^{43\text{Val}}$

Hb Ann Arbor: $\alpha_2\beta_2^{80\text{Arg}}$

Hb Genova: $\alpha_2\beta_2^{28\text{Pro}}$

Hb Shepherds Bush: $\alpha_2\beta_2^{74\text{Asp}}$

Hb Köln: $\alpha_2\beta_2^{98\text{Met}}$

Hb Wien: $\alpha_2\beta_2^{130\text{Asp}}$

c. Mild hemolysis: intermittent exacerbations

Hb Hasharon: $\alpha_2\beta_2^{47\text{His}}$

Hb Leiden: $\alpha_2\beta_2^{6}$ or 7 (Glu deleted)

Hb Freiburg: $\alpha_2\beta_2^{23}$ (Val deleted)

Hb Seattle: $\alpha_2\beta_2^{70\text{Asp}}$

Hb Louisville: $\alpha_2\beta_2^{42\text{Leu}}$

Hb Zurich: $\alpha_2\beta_2^{63\text{Arg}}$

Hb Gun Hill: $\alpha_2\beta_2^{91-95}$ (5 amino acids deleted)

d. No disease

Hb Etobicoke: $\alpha_2\beta_2^{84\text{Arg}}$

Hb Sogn: $\alpha_2\beta_2^{14\text{Arg}}$

Hb Tacoma: $\alpha_2\beta_2^{30\text{Ser}}$

2. Tetramers of normal chains; appear in thalassemias

Hb Bart: γ_4

Hb H: β_4

From Elghetany MT, Banki K: Erythrocyte disorders. In McPherson RA, Pincus MR: *Henry's clinical diagnosis and management by laboratory methods*, ed 22, Philadelphia, 2011, Elsevier, Saunders, p. 578. Originally modified from Winslow RM, Anderson WF: The hemoglobinopathies. In Stanbury JB, Wyngaarden JB, Fredrickson DS, et al, editors: *The metabolic basis of inherited disease*, ed 5, New York, 1983, McGraw-Hill, pp. 2281-2317. Updated from Patrinos GP, Giardine B, Riemer C, et al: Improvements in the HbVar database of human hemoglobin variants and thalassemia mutations for population and sequence variation studies, *Nucl Acids Res* 32 (database issue):D537-541, 2004. Available at: <http://globin.cse.psu.edu/hbvar/menu.html>. Accessed November 30, 2013.

Arg, Arginine; *Asn*, asparagine; *Asp*, aspartic acid; *Cys*, cysteine; *Gln*, glutamine; *Glu*, glutamic acid; *His*, histidine; *Leu*, leucine; *Lys*, lysine; *Met*, methionine; *Pro*, proline; *Ser* serine; *Thr*, threonine; *Tyr*, tyrosine; *Val*, valine.

occurring early in childhood between the time that passively acquired immunity dissipates and active immunity develops.¹⁰ In 1949, Pauling showed that when Hb S is subjected to electrophoresis, it migrates differently than does Hb A. This difference was shown to be caused by an amino acid substitution in the globin chain. Pauling and coworkers defined the genetics of the disorder and clearly distinguished heterozygous sickle trait (Hb AS) from the homozygous state (Hb SS).⁴

The term *sickle cell diseases* is used to describe a group of symptomatic hemoglobinopathies that have in common sickle cell formation and the associated crises. Patients with SCD are either homozygous for Hb S (SS) or are compound heterozygotes expressing Hb S in combination with another hemoglobin β chain mutation like Hb C or β -thalassemia. SCDs are the most common form of hemoglobinopathy, with Hb SS and the variants Hb SC and Hb S- β -thalassemia (Hb S- β -thal) occurring most frequently.

BOX 27-2 Clinically Important Hemoglobin (Hb)**Variants**

- I. Sickle syndromes
 - A. Sickle cell trait (AS)
 - B. Sickle cell disease
 1. SS
 2. SC
 3. SD-Punjab (Los Angeles)
 4. SO-Arab
 5. S- β -Thalassemia
 6. S-hereditary persistence of fetal hemoglobin
 7. SE
- II. Unstable hemoglobins→congenital Heinz body anemia (>140 variants)
- III. Hemoglobins with abnormal oxygen affinity
 - A. High affinity→familial erythrocytosis (>90 variants)
 - B. Low affinity→familial cyanosis (Hbs Kansas, Beth Israel, Yoshizuka, Agenogi, Titusville, Providence)
- IV. M hemoglobins→familial cyanosis (9 variants): Hb M-Boston, Hb M-Iwate, Hb Auckland, Hb Chile, Hb M-Saskatoon, Hb M-Milwaukee-1, Hb M-Milwaukee-2 (Hyde Park), Hb FM-Osaka, Hb FM-Fort Ripley
- V. Structural variants that result in a thalassemic phenotype
 - A. β -Thalassemia phenotype
 1. Hb Lepore ($\delta\beta$ fusion)
 2. Hb E
 3. Hb-Indianapolis, Hb-Showa-Yakushiji, Hb-Geneva
 - B. α -Thalassemia phenotype chain termination mutants (e.g., Hb Constant Spring)

Modified from Lukens JN: Abnormal hemoglobins: general principles (chap 39); Wong WC: Sickle cell anemia and other sickling syndromes (chap 40); Lukens JN: Unstable hemoglobin disease (chap 41). In Greer JP, Foerster J, Lukens JN, et al, editors: *Wintrobe's clinical hematology*, ed 11, Philadelphia, 2004, Lippincott Williams & Wilkins. Updated from Patrinos GP, Giardine B, Riemer C, et al: Improvements in the HbVar database of human hemoglobin variants and thalassemia mutations for population and sequence variation studies, *Nucl Acids Res* 32(database issue):D537-541, 2004. Available at: <http://globin.cse.psu.edu/hbvar/menu.html>. Accessed November 30, 2013.

Inheritance Pattern

As stated earlier, the genes that code for the globin chains are located at specific loci on chromosomes 16 and 11. The α -like genes (α and ζ) are located on the short arm of chromosome 16, whereas the β -like genes (β , γ , δ , and ϵ) are located on the short arm of chromosome 11. With the exception of the γ genes, which have four loci, each β -like gene has two loci. β -hemoglobin variants are inherited as autosomal codominants, with one gene inherited from each parent.⁴

Patients with SCD (Hb SS), Hb SC, or Hb S- β -thal have inherited a sickle (S) gene from one parent and an S, C, or β -thalassemia gene from the other. Among patients with SCD, individuals who are homozygotes (Hb SS) have more severe disease than individuals who are compound heterozygotes for Hb S (Hb SC or Hb S- β -thal). Heterozygotes (Hb AS) are generally asymptomatic. Using Hb S and Hb C as examples,

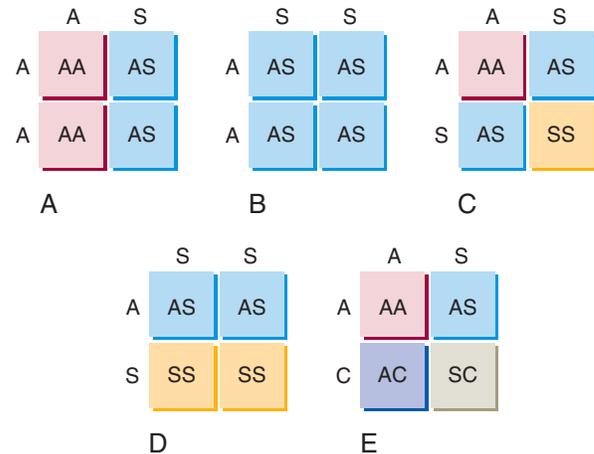


Figure 27-2 Punnett square illustrating the standard method for predicting the inheritance of abnormal hemoglobins. Each parent contributes one gene.

Figure 27-2 illustrates the inheritance of abnormal hemoglobins involving mutations in the β gene.

Prevalence

The highest frequency of the sickle cell gene is found in sub-Saharan Africa, where each year approximately 230,000 babies are born with sickle cell disease (Hb SS), representing 0.74% of all live births occurring in this area.¹¹ In contrast, approximately 2600 babies are born annually with sickle cell disease in North America and 1300 in Europe.¹¹ Globally, the sickle cell gene occurs at the highest frequency in five geographic areas: sub-Saharan Africa, Arab-India, the Americas, Eurasia, and Southeast Asia. In 2010, these five geographic areas accounted for 64.4%, 22.7%, 7.4%, 5.4%, and 0.1%, respectively, of all neonates born globally with sickle cell trait, and 75.5%, 16.9%, 4.6%, 3.0%, and 0%, respectively, of all neonates born globally with sickle cell disease. Three countries accounted for approximately 50% of neonates with SS and AS genotypes: Nigeria, India, and DR Congo.¹² Although in the United States, SCD is found mostly in individuals of African descent, it also has been found in individuals from the Middle East, India, and the Mediterranean area (Figure 27-3). SCD can also be found in individuals from the Caribbean and Central and South America.¹³ The sickle cell mutation is becoming more prominent in southern India, particularly in certain tribes.¹⁴ It is estimated that 25,000 babies are born annually with sickle cell anemia in India.²

Etiology and Pathophysiology

Hb S is defined by the structural formula $\alpha_2\beta_2^{6\text{Glu}\rightarrow\text{Val}}$, which indicates that on the β chain at position 6, glutamic acid is replaced by valine. The mutation occurs in nucleotide 17, where thymine is changed to adenine, resulting in a change in codon 6 and the substitution of valine for glutamic acid at amino acid position 6.¹¹ Glutamic acid has a net charge of (-1), whereas valine has a net charge of (0). This amino acid substitution produces a change in charge of (+1), which affects the electrophoretic mobility of the hemoglobin molecule. This amino acid substitution also affects the way the hemoglobin molecules interact with one another within the erythrocyte

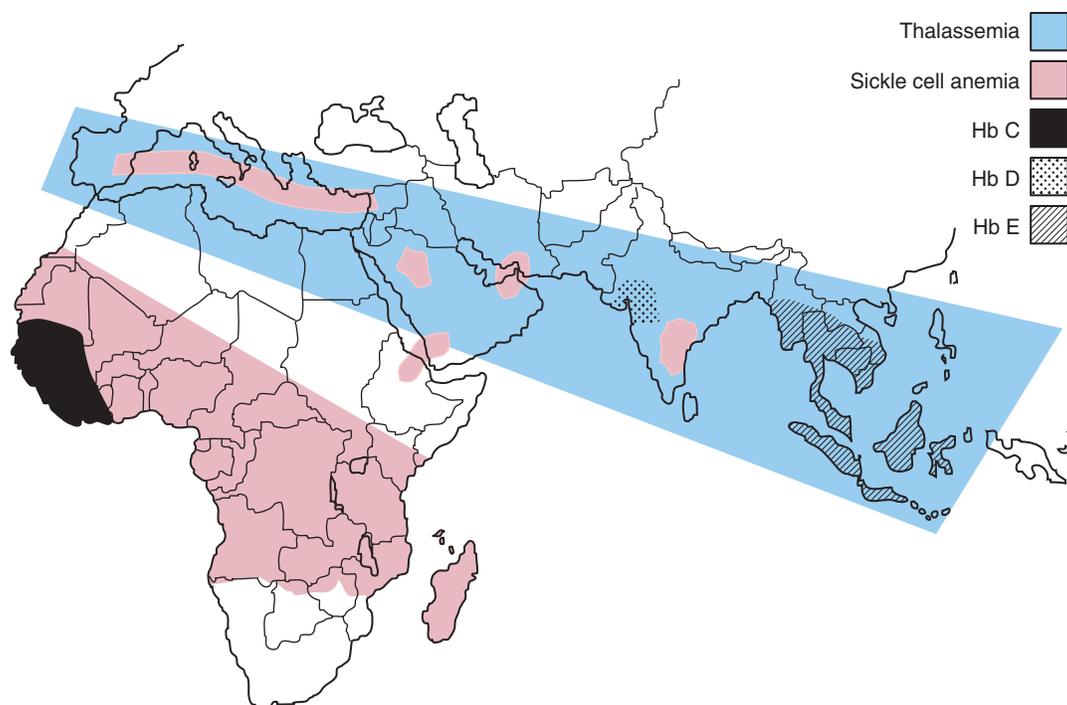


Figure 27-3 Geographic distribution of common inherited structural hemoglobin variants and the thalassemias. (From Hoffbrand AV, Pettit JE: *Essential haematology*, ed 3, Oxford, 1993, Blackwell Scientific.)

cytosol. The nonpolar (hydrophobic) valine amino acid has been placed in the position that the polar glutamic acid once held. Because glutamic acid is polar, the β chain folds in such a way that glutamic acid extends outward from the surface of the hemoglobin tetramer to bind water and contribute to hemoglobin solubility in the cytosol. Therefore, the hydrophobic valine is also extended outward, but instead of binding water, it seeks a hydrophobic niche with which to bind. When Hb S is fully oxygenated, the quaternary structure of the molecule does not produce a hydrophobic pocket for valine to bind to, which allows the hemoglobin molecules to remain soluble in the erythrocyte cytosol like Hb A and maintains the normal biconcave disc shape of the RBCs. However, the natural allosteric change that occurs upon deoxygenation creates a hydrophobic pocket in the area of phenylalanine 85 and leucine 88, which allows the valine from an adjacent hemoglobin molecule to bind. This hemoglobin pairing creates an orientation that helps other hemoglobin molecules to form electrostatic bonds between amino acids and becomes the seed for polymer formation. Other hemoglobin pairs polymerize, forming a hemoglobin core composed of four hemoglobin molecules that elongate in a helical formation. An outer layer of 10 hemoglobin molecules forms around the 4-hemoglobin-molecule core, creating the long, slender Hb S polymer.¹⁵⁻¹⁸ Hb S molecules within the RBCs become less soluble, forming tactoids or liquid crystals of Hb S polymers that grow in length beyond the diameter of the RBC, causing sickling. In homozygotes, the sickling process begins when oxygen saturation decreases to less than 85%. In heterozygotes, sickling does not occur unless the oxygen saturation of hemoglobin is reduced to less than 40%.¹⁹ The blood becomes more viscous when polymers are formed and sickle cells are created.¹⁹ Increased blood viscosity

and sickle cell formation slow blood flow. In addition to a decrease in oxygen tension, there is a reduction in the pH and an increase in 2,3-bisphosphoglycerate. Reduced blood flow prolongs the exposure of Hb S-containing erythrocytes to a hypoxic environment, and the lower tissue pH decreases the oxygen affinity, which further promotes sickling. The end result is occlusion of capillaries and arterioles by sickled RBCs and infarction of surrounding tissue.

Sickle cells occur in two forms: reversible sickle cells and irreversible sickle cells.²⁰ Reversible sickle cells are Hb S-containing erythrocytes that change shape in response to oxygen tension. Reversible sickle cells circulate as normal biconcave discs when fully oxygenated but undergo hemoglobin polymerization, show increased viscosity, and change shape on deoxygenation. The vasoocclusive complications of SCD are thought to be due to reversible sickle cells that are able to travel into the microvasculature in the biconcave disk conformation due to their normal rheologic properties when oxygenated and then become distorted and viscous as they become deoxygenated, converting to the sickle cell configuration in the vessel.

In contrast, irreversible sickle cells do not change their shape regardless of the change in oxygen tension or degree of hemoglobin polymerization. These cells are seen on the peripheral blood film as elongated sickle cells with a point at each end. It is thought that irreversible sickle cells are recognized as abnormal by the spleen and removed from circulation, which prevents them from entering the microcirculation and causing vasoocclusion.

Not only the oxygen tension but also the level of intracellular hydration affects the sickling process. When RBCs containing Hb S are exposed to a low oxygen tension, hemoglobin polymerization occurs. Polymerized deoxyhemoglobin S activates a

membrane channel called P_{sickle} that is otherwise inactive in normal RBCs. These membrane channels open when the blood partial pressure of oxygen decreases to less than 50 mm Hg. Open P_{sickle} channels allow the influx of Ca^{2+} , raising the intracellular calcium levels and activating a second membrane channel called the *Gardos channel*. An activated Gardos channel causes the efflux of K^+ , which stimulates the efflux of Cl^- through another membrane channel to maintain charge equilibrium across the RBC membrane. The efflux of these ions leads to water efflux and intracellular dehydration, effectively increasing the intracellular concentration of Hb S and intensifying polymerization. Another contributor to K^+ and Cl^- efflux and the resultant dehydration is the K^+/Cl^- cotransporter system. Ironically, this system is activated by dehydration and positively charged hemoglobins such as Hb S and Hb C. The K^+/Cl^- cotransporter pathway is also activated by the low pH encountered in the spleen and kidneys. One potential explanation for the altered function of the membrane channels is oxidative damage triggered by Hb S polymerization. Injury to the RBC membrane induces adherence to endothelial surfaces, which causes RBC aggregation, produces ischemia, and exacerbates Hb S polymerization.¹⁰

Another important factor in the pathophysiology of SCD involves the redistribution of phospholipids in the RBC membrane, which contributes to hemolysis, vasoocclusive crisis, stroke, and acute chest syndrome. In the bilayer membranes of normal RBCs, choline phospholipids like sphingomyelin and phosphatidylcholine are located on the outer plasma layer, whereas aminophospholipids like phosphatidylserine (PS) and phosphatidylethanolamine are primarily on the inner cytoplasmic layer of the membrane. This asymmetrical distribution of

membrane phospholipids is accomplished by adenosine triphosphate–dependent enzymes called *translocases* or *flippases*. Inhibition of flippases and activation of an enzyme called *scramblase* cause a more random distribution of membrane phospholipids, which increases the number of choline phospholipids on the interior half of the membrane and the number of aminophospholipids on the exterior membrane surface. The sickle cells of homozygotes (Hb SS) express 2.1% PS on erythrocyte exterior surfaces compared with 0.2% for normal Hb AA controls.^{21,22} It is hypothesized that Hb S polymerization may produce microparticles and iron complexes that adhere to the RBC membrane and generate reactive oxygen species, which, along with increased intracellular calcium or protein kinase C activation, may contribute to flippase inhibition and scramblase activation.^{23,24} PS on the exterior surface of RBCs binds thrombospondin on vascular endothelial cells,²⁵ enhancing adherence between RBCs and the vessel wall and contributing to vasoocclusive crisis, activation of coagulation, and decreased RBC survival.^{26,27} In addition, RBCs with PS on the external membrane surface are vulnerable to hydrolysis by secretory phospholipase A_2 (sPLA₂), which generates lysophospholipids and fatty acids like lysophosphatidic acid. This results in vascular damage that contributes to acute chest syndrome.^{28,29}

Clinical Features

The clinical manifestations of SCD can vary from no symptoms to a potentially lethal state. Symptoms also vary between ethnic groups with Indian patients expressing a much milder disease than their African counterparts.¹⁴ People with SCD can develop a variety of symptoms as listed in [Box 27-3](#). Over a

BOX 27-3 Clinical Features of Sickle Cell Disease

I. Vasoocclusion

A. Causes:

- Acidosis
- Hypoxia
- Dehydration
- Infection
- Fever
- Extreme cold

B. Clinical manifestations

1. Bones:
 - Pain
 - Hand-foot dactylitis
 - Infection (osteomyelitis)
2. Lungs:
 - Pneumonia
 - Acute chest syndrome
3. Liver:
 - Hepatomegaly
 - Jaundice
4. Spleen:
 - Sequestration splenomegaly
 - Autosplenectomy

5. Penis:

- Priapism

6. Eyes:

- Retinal hemorrhage

7. Central nervous system

8. Urinary tract:

- Renal papillary necrosis

9. Leg ulcers

II. Bacterial infections

- A. Sepsis
- B. Pneumonia
- C. Osteomyelitis

III. Hematologic defects

- A. Chronic hemolytic anemia
- B. Megaloblastic episodes
- C. Aplastic episodes

IV. Cardiac defects

- A. Enlarged heart
- B. Heart murmurs

V. Other clinical features

- A. Stunted growth
- B. High-risk pregnancy

adults with acute chest syndrome die from complications linked to chronic lung disease and pulmonary hypertension.³⁸ In children, acute chest syndrome generally is precipitated by infection characterized by fever, cough, and tachypnea. Acute chest syndrome is also linked with sPLA₂, discussed previously. The level of sPLA₂ has been shown to be a predictor of acute chest syndrome in patients with SCD³⁹ in that sPLA₂ rises 24 to 48 hours before symptoms of acute chest syndrome begin.⁴⁰ In addition, a high sPLA₂ level correlates with the degree of lung damage.

Pulmonary hypertension (PHT) is a serious and potentially fatal sequela of SCD. Among patients with SCD, PHT has a prevalence of about 33%, with 10% of patients manifesting a more severe version.⁴¹ The mortality rate for sickle cell patients who develop PHT is 40% at 40 months.⁴¹ An association has been documented between the development of PHT and the nitrous oxide (NO) pathway. NO is produced from the action of endothelial NO synthase (eNOS) on arginine, which causes vasodilation. Patients with SCD have a decrease in NO, and this leads to vasoconstriction and hypertension.^{4,41} In addition, low NO levels in the blood fail to inhibit endothelin-1, a potent vasoconstrictor, which results in additional vasoconstriction and hypertension.³⁸ The connection between NO and SCD involves the hemolytic crisis. Erythrocyte hemolysis releases high levels of arginase, which degrades arginine; the result is less NO production from eNOS.^{42,43} In addition, the free hemoglobin released from hemolyzed RBCs scavenges NO, which further reduces the levels and exacerbates the vasoconstriction and hypertension.⁴¹ Blood arginine and NO levels drop a few days before the onset of acute chest syndrome,³⁷ a finding suggesting that the NO pathway is a connection between SCD, PHT, and asthma.^{38,44} Treatment with large doses of arginine reduces pulmonary artery pressure, but the effect is not sustainable and does not reduce mortality. An increased tricuspid regurgitation velocity (TRV) and blood NT-ProBNP levels above 160 ng/L were found to be good predictors of pulmonary hypertension and are associated with a higher mortality rate.³³ Bosentan is the treatment of choice for pulmonary hypertension, but liver enzymes should be monitored for liver toxicity.³³

Bacterial infections pose a major problem for SCD patients. These patients have increased susceptibility to life-threatening infection from *Staphylococcus aureus*, *Streptococcus pneumoniae*, and *Haemophilus influenzae*. Acute infections are common causes of hospitalization and have been the most frequent cause of death, especially in the first 3 years of life.¹ Bacterial infections of the blood (septicemia) are exacerbated by the autosplenectomy effect as the spleen gradually loses its ability to function as a secondary lymphoid tissue to effectively clear organisms from the blood.

Chronic hemolytic anemia is characterized by shortened RBC survival of between 16 and 20 days,⁴⁵ with a corresponding decrease in hemoglobin and hematocrit, an elevated reticulocyte count, and jaundice. Continuous screening and removal of sickle cells by the spleen perpetuate the chronic hemolytic anemia and autosplenectomy effect. Because other conditions, such as hepatitis and gallstones, may cause jaundice, chronic hemolysis is difficult to diagnose in sickle cell

patients.²¹ RBC hemolysis releases free hemoglobin, which disrupts the arginine-nitric oxide pathway, resulting in the sequestration and lowering of nitric oxide.^{31,45} Decreased NO leads to endothelial cell activation, vasoconstriction, adherence of RBCs to the endothelium, and pulmonary hypertension previously discussed.⁴⁵ Another major sequelae of hemolysis is renal dysfunction, which can be detected early by an increased glomerular filtration rate of 140 mL/min per 1.73 m³ found in 71% of patients with SCD.⁴⁶ Progression of renal dysfunction can be identified by detecting microalbuminuria (>4.5 mg/mmol), followed by proteinuria and terminating in elevated BUN and creatinine levels. Angiotensin-converting enzyme inhibitors (ACEI) have been shown to lower proteinuria in SCD patients.³³

Megaloblastic episodes result from the sudden arrest of erythropoiesis due to folate depletion. Folic acid deficiency as a cause of exaggerated anemia in SCD is extremely rare in the United States. It is common practice to prescribe prophylactic folic acid for patients with SCD, however.⁸

Aplastic episodes (bone marrow failure) are the most common life-threatening hematologic complications and are usually associated with infection, particularly parvovirus infection.³⁴ Aplastic episodes present clinical problems similar to those seen with other hemolytic disorders.⁴⁷ Sickle cell patients usually can compensate for the decrease in RBC survival by increasing bone marrow output. When the bone marrow is suppressed temporarily by bacterial or viral infections, however, the hematocrit decreases substantially with no reticulocyte compensation. The spontaneous recovery phase is characterized by the presence of nucleated RBCs and an increase in the number of reticulocytes in the peripheral blood. Most aplastic episodes are short-lived and require no therapy. If anemia is severe and the bone marrow remains aplastic, transfusions become necessary. If patients are not transfused in a timely fashion, death can occur.⁴⁷

Patients also experience cardiac defects, including enlarged heart and heart murmurs. In patients with severe anemia, cardiomegaly can develop as the heart works harder to maintain adequate blood flow and tissue oxygenation. Increased cardiac workload along with increased bone marrow erythropoiesis increases calorie burning, contributing to a reduced growth rate.⁴⁸ When patients enter childbearing age, pregnancy becomes risky.⁴

Impaired blood supply to the head of the femur and humerus results in a condition called *avascular necrosis* (AVN). About 50% of patients with SCD develop AVN by 35 years of age.⁴⁹ Physical therapy and surgery to relieve intramedullary pressure within the head of the long bones are effective, but hip and/or shoulder implants become necessary in most patients experiencing AVN.⁴⁹ Similarly, leg ulcers are a common complication of SCD. Ulcers tend to heal slowly, develop unstable scars, and recur at the same site, becoming a chronic problem, with associated chronic pain.⁴⁷

Microstrokes can lead to headaches, poor school performance, reduced intelligence quotient (IQ), and overt central nervous system dysfunction. A neurologic examination followed by magnetic resonance imaging and, if available, transcranial

Doppler ultrasonography or magnetic resonance angiography is recommended to detect microstrokes.⁴⁸

Incidence with Malaria and Glucose-6-Phosphate Dehydrogenase Deficiency

The sickle gene occurs with greatest frequency in Central Africa, the Near East, the region around the Mediterranean, and parts of India. The frequency of the gene parallels the incidence of *P. falciparum* and seems to offer some protection against cerebral falciparum malaria in young patients. Malarial parasites are living organisms within the RBCs that use the oxygen within the cells. This reduced oxygen tension causes the cells to sickle, which results in injury to the cells. These injured cells tend to become trapped within the blood vessels of the spleen and other organs, where they are easily phagocytized by scavenger WBCs. Selective destruction of RBCs containing parasites decreases the number of malarial organisms and increases the time for immunity to develop. One explanation for this phenomenon is that the infected cell is uniquely sickled and destroyed, probably in an area of the spleen or liver, where phagocytic cells are plentiful, and the oxygen tension is significantly decreased.⁵⁰

Because of the high incidence of glucose-6-phosphate dehydrogenase (G6PD) deficiency in patients with SCD, it has been suggested that G6PD deficiency has a protective effect in these patients,⁵¹ although this correlation has not been confirmed through studies. It also has been postulated that hemolytic episodes are more common in these patients. In the first 42 months of life, patients with SCD and G6PD deficiency had lower steady-state hemoglobin levels, higher reticulocyte counts, three times more acute anemia events, and more frequent blood transfusions—vasoocclusive and infectious events than matched sickle cell patients without G6PD deficiency.³⁰ Because of the presence of young cells rich in G6PD, however, the increased hemolysis is more likely caused by the enzyme abnormality when the population is shifted to the oldest cells during an aplastic crisis.⁵²

Laboratory Diagnosis

The anemia of SCD is a chronic hemolytic anemia, classified morphologically as normocytic, normochromic. The characteristic diagnostic cell observed on a Wright-stained peripheral blood film is a long, curved cell with a point at each end (Figure 27-5). Because of its appearance, the cell was named a *sickle cell*.³² The peripheral blood film shows marked poikilocytosis and anisocytosis with normal RBCs, sickle cells, target cells, nucleated RBCs along with a few spherocytes, basophilic stippling, Pappenheimer bodies, and Howell-Jolly bodies. The presence of sickle cells and target cells is the hallmark of SCD. There is moderate to marked polychromasia with a reticulocyte count between 10% and 25%, corresponding with the hemolytic state and the resultant bone marrow response. The RBC distribution width (RDW) is increased owing to moderate anisocytosis. The mean cell volume (MCV) is not as elevated as one would expect, however, given the elevated reticulocyte count. An aplastic crisis can be heralded by a decreased reticulocyte count. Moderate leukocytosis is usually present (sometimes 40 to 50×10^9 WBC/L) with neutrophilia and a mild shift toward immature granulocytes. The leukocyte alkaline phosphatase score is not elevated when neutrophilia is caused by sickle cell crisis alone when no underlying infection is present. Thrombocytosis is usually present. The bone marrow shows erythroid hyperplasia, reflecting an attempt to compensate for the anemia, which results in polychromasia and an increase in reticulocytes and nucleated RBCs in the peripheral blood. Levels of immunoglobulins, particularly immunoglobulin A, are elevated in all forms of SCD. Serum ferritin levels are normal in young patients but tend to be elevated later in life; however, hemochromatosis is rare. Chronic hemolysis is evidenced by elevated levels of indirect and total bilirubin with the accompanying jaundice.

The diagnosis of SCD is generally a two-step process by first demonstrating the insolubility of deoxygenated Hb S in solution followed by confirmation of its presence using hemoglobin electrophoresis, high-performance liquid chromatography (HPLC), or capillary electrophoresis. For more complicated

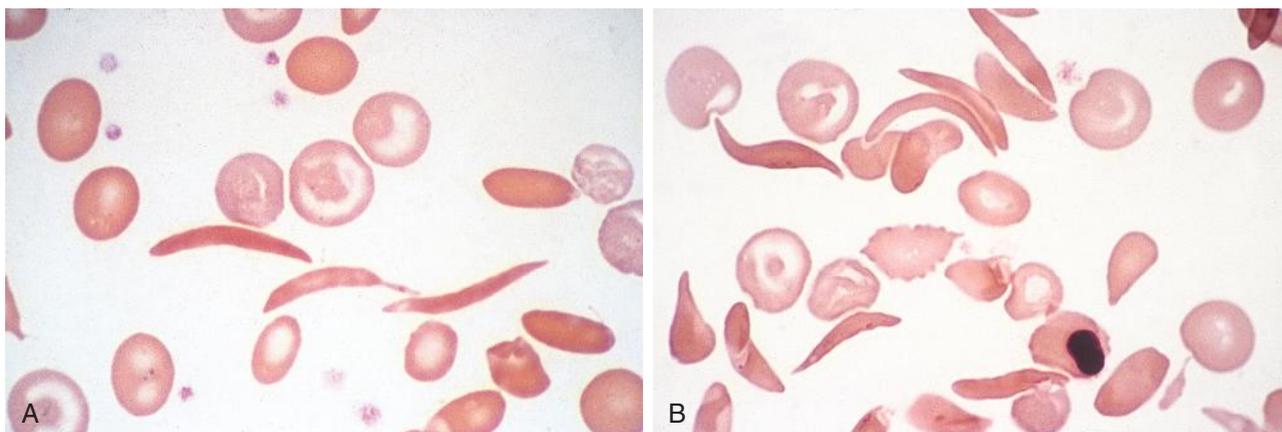


Figure 27-5 **A**, Peripheral blood film for a patient with sickle cell disease (SCD) showing anisocytosis, polychromasia, three sickle cells, target cells, and normal platelets ($\times 1000$). **B**, Peripheral blood film for an SCD patient showing anisocytosis, poikilocytosis, sickle cells, target cells, and one nucleated RBC ($\times 1000$). Platelets are not present in this field, but their numbers were adequate in this patient. (Courtesy Ann Bell, University of Tennessee, Memphis.)

cases, isoelectric focusing, tandem mass spectrometry, or DNA analysis may be needed. An older screening test detects Hb S insolubility by inducing sickle cell formation on a glass slide. A drop of blood is mixed with a drop of 2% sodium metabisulfite (a reducing agent) on a slide, and the mixture is sealed under a coverslip. The hemoglobin inside the RBCs is reduced to the deoxygenated form; this induces polymerization and the resultant sickle cell formation, which can be identified microscopically. This method is slow and cumbersome and is rarely used.

The most common screening test for Hb S, called the hemoglobin solubility test, capitalizes on the decreased solubility of deoxygenated Hb S in solution, producing turbidity. Blood is added to a buffered salt solution containing a reducing agent, such as sodium hydrosulfite (dithionite), and a detergent-based lysing agent (saponin). The saponin dissolves membrane lipids, causing the release of hemoglobin from the RBCs, and the dithionite reduces the iron from the ferrous to the ferric oxidation state. Ferric iron is unable to bind oxygen, converting the hemoglobin to the deoxygenated form. Deoxygenated Hb S polymerizes in solution, which renders it turbid, whereas solutions containing non-sickling hemoglobins remain clear (Figure 27-6). False-positive results for Hb S can occur with hyperlipidemia, a few rare hemoglobinopathies, and when too much blood is added to the test solution; false-negative results can occur in infants less than 6 months of age and with low hematocrits. Other hemoglobins that give a positive result on the solubility test include Hb C-Harlem (Georgetown), Hb C-Ziguinchor, Hb S-Memphis, Hb S-Travis, Hb S-Antilles, Hb S-Providence, Hb S-Oman, Hb Alexander, and Hb Porte-Alegre.^{4,8} All of these hemoglobins have two amino acid substitutions: the Hb S substitution ($\beta^{6\text{Glu}\rightarrow\text{Val}}$) and another

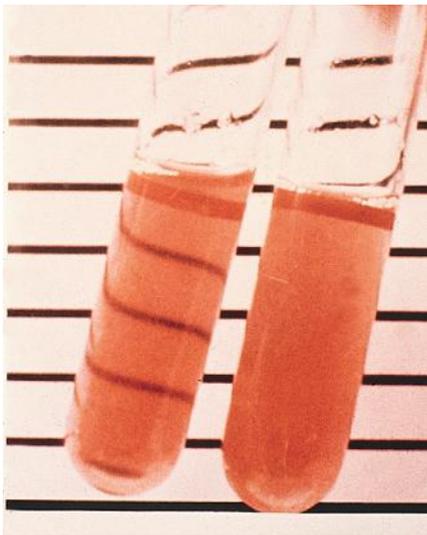


Figure 27-6 Tube solubility screening test for the presence of hemoglobin S. In a negative test result (*left*), the solution is clear and the lines behind the tube are visible. In a positive test result (*right*), the solution is turbid and the lines are not visible. (Courtesy Ann Bell, University of Tennessee, Memphis.)

unrelated substitution. Hb S-Antilles is particularly important because it can cause sickling in the heterozygous state.

Alkaline hemoglobin electrophoresis is a common first step in the confirmation of hemoglobinopathies, including SCD. Electrophoresis is based on the separation of hemoglobin molecules in an electric field due primarily to differences in total molecular charge. In alkaline electrophoresis, hemoglobin molecules assume a negative charge and migrate toward the anode (positive pole). Historically, alkaline hemoglobin electrophoresis was performed on cellulose acetate medium but is being replaced by electrophoresis on agarose medium. Nonetheless, because some hemoglobins have the same charge and, therefore, the same electrophoretic mobility patterns, hemoglobins that exhibit an abnormal electrophoretic pattern at an alkaline pH may be subjected to electrophoresis at an acid pH for definitive separation. In an acid pH some hemoglobins assume a negative charge and migrate toward the anode, while others are positively charged and migrate toward the cathode (negative pole). For example, Hb S migrates with Hb D and Hb G on alkaline electrophoresis but separates from Hb D and Hb G on acid electrophoresis. Similarly, Hb C migrates with Hb E and Hb O on alkaline electrophoresis but separates on acid electrophoresis. Figure 27-7 shows electrophoretic patterns for

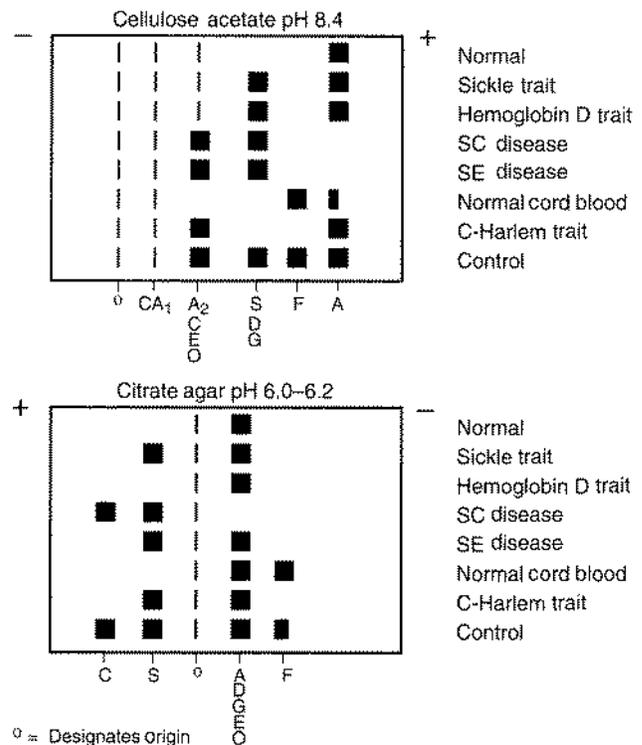


Figure 27-7 Relative mobilities of normal and variant hemoglobins in various conditions measured by electrophoresis on cellulose acetate at an alkaline pH and citrate agar at an acid pH. The relative amount of hemoglobin is not proportional to the size of the band; for example, in sickle cell trait (Hb AS), the bands may appear equal, but the amount of Hb A exceeds that of Hb S. (From Schmidt RM, Brosious EF: *Basic laboratory methods of hemoglobinopathy detection*, ed 6, HEW Pub No (CDC) 77-8266, Atlanta, 1976, Centers for Disease Control and Prevention.)

normal and abnormal hemoglobins. Figure 27-8 shows the electrophoretic separation of a normal adult and a patient with sickle cell disease (Hb SS) at an alkaline pH. Figure 27-9 shows the HPLC and capillary electrophoresis are gaining in popularity because these methods are more automated, the instruments are more user friendly, and they can be used to confirm hemoglobin variants observed with electrophoresis (Figure 27-9).

HPLC separates hemoglobin types in a cation exchange column and usually requires only one sample injection. Unlike electrophoresis, HPLC can identify and quantitate low levels of Hb A₂ and Hb F, but comigration of Hb A₂ and Hb E occurs. Therefore, HPLC is best used in the diagnosis of thalassemias rather than hemoglobinopathies because quantitation of low levels of normal and abnormal hemoglobin levels is necessary to distinguish thalassemias. HPLC is also commonly used to quantitate Hb A1c levels to monitor diabetic patients.

Capillary electrophoresis, like agarose electrophoresis, separates hemoglobin types based on charge in an alkaline buffer but does so using smaller volumes and produces better separation than traditional agarose electrophoresis. Semiautomated systems like the Capillars[®] system (Sebia, Evry, France) allow for the testing of up to eight samples in parallel with computerized analysis of results. Capillary electrophoresis is also economical, since each capillary can accommodate at least 3000 runs.¹ In 2009 hemoglobin electrophoresis in agarose medium was still the most commonly used technique to identify hemoglobin variants, but capillary electrophoresis and HPLC are gaining in popularity.⁵³

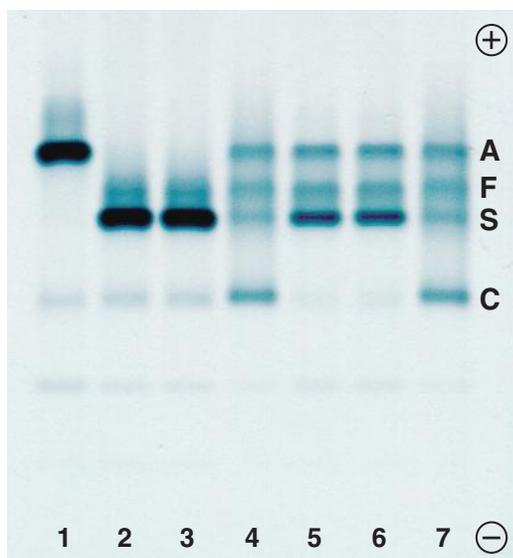


Figure 27-8 Electrophoretic separation of hemoglobins (Hb) at alkaline pH. **1**, Normal adult; **2** and **3**, 17-year-old patient with sickle cell anemia (Hb SS); **5** and **6**, patient with sickle cell anemia, recently transfused (note the presence of Hb A from the transfused red blood cells); **4** and **7**, Hbs A/F/S/C standard (Hydrigel 7 Hemoglobin/Hydrasys System, Sebia Electrophoresis, Norcross, GA). (Modified from Elghetany MT, Banki K: Erythrocytic disorders. In McPherson RA, Pincus MR: *Henry's Clinical Diagnosis and Management by Laboratory Methods*, ed 22, Philadelphia, 2011, Elsevier, p. 578.)

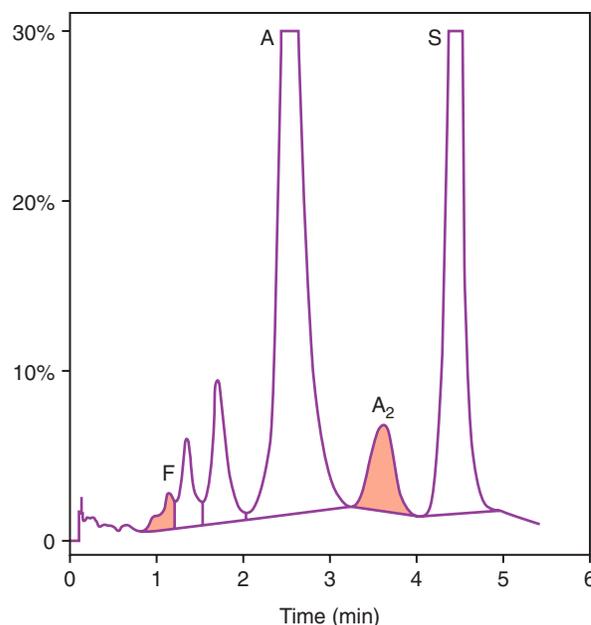


Figure 27-9 Ion-exchange high-performance liquid chromatography (HPLC) separation of hemoglobins (Hbs) in a patient with sickle cell trait demonstrating Hbs F, A, A₂, and an abnormal Hb in the S window (Bio-Rad Variant Classic Hb Testing System, BioRad Laboratories, Philadelphia). (Modified from Elghetany MT, Banki K: Erythrocytic disorders. In McPherson RA, Pincus MR: *Henry's Clinical Diagnosis and Management by Laboratory Methods*, ed 22, Philadelphia, 2011, Elsevier, p. 578.)

Isoelectric focusing (IEF) is a confirmatory technique that is expensive and complex, requiring well-trained and experienced laboratory personnel. The method uses an electric current to push the hemoglobin molecules across a pH gradient. The charge of the molecules change as they migrate through the pH gradient until the hemoglobin species reaches its isoelectric point (net charge of zero). With a net charge of zero, migration stops and the hemoglobin molecules accumulate at their isoelectric position. Molecules with isoelectric point differences of as little as 0.02 pH units can be effectively separated.¹

Neonatal screening requires a more sophisticated approach, often using three techniques: adapted IEF, HPLC, and reversed-phase HPLC. This multisystem approach is needed to distinguish not only the multitude of hemoglobin variants but also the numerous thalassemias. The more progressive laboratories use a combination of two or more techniques to improve identification of hemoglobin variants. Some reference laboratories may use mass spectroscopy, matrix-assisted laser desorption-ionization time-of-flight (MALDI-TOF) mass spectrometry, or isoelectric focusing to separate hemoglobin types, or nucleic acid identification of the genetic mutation.^{1,54}

Patients with Hb SS or Hb SC disease lack normal β -globin chains, so they have no Hb A. In Hb SS, the Hb S level is usually greater than 80%. The Hb F level is usually increased (1% to 20%), and when Hb F constitutes more than 20% of hemoglobin, it has a tendency to modulate the severity of the disease. This is especially true in newborns and in

patients with hereditary persistence of fetal hemoglobin.³¹ The Hb A₂ level is normal or slightly increased (2% to 5%), and Hb A₂ quantitation is useful in differentiating Hb SS from Hb S-β⁰-thalassemia, in which Hb A₂ is increased (Chapter 28). Hb G, Hb D, and Hb S all migrate in the same position on alkaline cellulose acetate and alkaline agarose electrophoresis, but Hb G and Hb D do not give a positive result on the tube solubility test.

The typical sequelae of SCD may be predicted, and the effectiveness of treatment monitored, if reliable biomarkers of inflammation can be identified. Among the common indicators of inflammation, WBC is a good predictor of sickle cell events and mortality, whereas the erythrocyte sedimentation rate and C-reactive protein (CRP) level exhibit variability too great to reliably predict events. However, CRP and sPLA₂ are both elevated during vasoocclusive crisis and acute chest syndrome. Other markers such as interleukin-6 (IL-6), IL-10, and protein S are showing promise as useful indicators in clinical practice.⁵⁵ Annexin A5, a protein bound to lipids in the plasma membrane of endothelial cells and platelets, has been shown to elevate before and during VOC.⁵⁶ Lipid damage from oxidative stress can be predicted by plasma elevations of malondialdehyde (MDA) and depleted α-tocopherol. In addition, α-tocopherol rises with CRP during bouts of inflammation. Of all the biomarkers evaluated, IL-6, IL-10, vascular cell adhesion molecule 1 (VCAM-1), and sPLA₂ are the most promising at predicting impending crisis.⁵⁷

Treatment

Supportive care has been the mainstay of therapy for SCD. New therapies have evolved, however, that are actually modifying the genetic pathogenesis of the disease. Neonatal screening, childhood prophylactic penicillin therapy, bone marrow transplantation, and treatment with hydroxyurea (hydroxycarbamide) in adults may extend the life of the SCD patient further.

The main components of supportive therapy include adequate hydration, prophylactic vitamin therapy, avoidance of low-oxygen environments, analgesia for pain, and aggressive antibiotic therapy with the first signs of infection. Hydration maintains good blood flow and reduces vasoocclusive crises. Prophylactic oral penicillin V at a dose of 125 mg twice per day by the age of 3 months to 3 years of age is recommended to avoid infection and the associated morbidity and mortality. The penicillin dosage is increased to 250 mg twice per day from 3 to 5 years of age.⁵⁸ When infections occur, prompt antibiotic treatment reduces the associated morbidity and mortality.⁹ Avoidance of strenuous exercise, high altitudes, and unpressurized air travel maintains high oxygen tensions and reduces the sickling phenomenon. Treatment for painful episodes includes ensuring optimal hydration, rapidly treating associated infection, oxygen therapy, and effectively relieving pain. Analgesics are the foundation of pain management, with nonsteroidal antiinflammatory drugs like paracetamol (acetaminophen) and nefopam administered to manage mild ischemic attacks. Opioids like meperidine (pethidine or demerol) or tramadol are recommended when pain becomes chronic.⁵⁹

Acute VOC attacks are often treated with morphine in the emergency department or when in transit. Blood exchange transfusion (BET) is the treatment of choice for severe VOC attacks and acute chest syndrome (ACS).³³ Painful crises tend to increase with age, and physicians must be aware of opiate tolerance and rebound pain following opiate therapy, called central sensitization. Repeated painful crises can result in hypersensitivity to repeated pain by increasing peripheral inflammation, increased neurotransmitter release, increased calcium influx into postsynaptic junctions, and other pathways that increase pain signals to the brain.⁴⁵ This phenomenon can be misinterpreted as drug intolerance, causing inappropriate dose escalation, or as drug-seeking behavior, causing inappropriate termination of treatment. The most appropriate response to opioid tolerance and central sensitization is a gradual dose reduction to reset the pain receptors followed by switching to opioids such as methadone and buprenorphine that are less sensitive to this phenomenon.⁴⁵ The patient should be examined on a regular basis, and routine testing should be done to establish baseline values for the patient during nonsickling periods.

Children younger than 3 years often experience *hand-foot syndrome*, characterized by pain and swelling in the hands and feet.³³ Treatment usually consists of increasing intake of fluids and giving analgesics for pain.

Pneumococcal disease has been a leading cause of morbidity and mortality in children, especially children younger than 6 years. With immunization and prophylactic antibiotics, however, this is now a preventable complication.⁷ Immunization with heptavalent conjugated pneumococcal vaccine is recommended at 2, 4, and 6 months of age. The 23-valent pneumococcal vaccine is recommended at 2 years, with a booster at the age of 5 years. Standard childhood vaccinations should be given as scheduled. In addition, annual administration of influenza vaccine is recommended beginning at 6 months of age.⁴² The risk of bacterial infection probably increases in mature patients with Hb SC disease and homozygous SCD.²⁰

Transfusions can be used to prevent the complications of SCD. More specifically, periodic transfusions, given at a frequency of eight or more per year, are effective at preventing stroke, symptomatic anemia, brain injury, priapism, leg ulcers, PHT, delayed pubescence, splenomegaly, and chronic pain and improving school attendance, IQ, energy, exercise tolerance, mood, and sense of well-being. In other circumstances, such as central nervous system infarction, hypoxia with infection, stroke, episodes of acute chest syndrome, and preparation for surgery, transfusions are used to decrease blood viscosity and the percentage of circulating sickle cells. Before all but simple surgeries, Hb SS patients are transfused with normal Hb AA blood to bring the volume of Hb S to less than 50% or to achieve a hemoglobin of 10 g/dL in an effort to prevent complications in surgery.^{58,60} Maintenance transfusions should be given in pregnancy if the mother experiences vasoocclusive or anemia-related problems or if there are signs of fetal distress or poor growth.²⁰ Nonetheless, transfusion therapy has the potential to cause transfusion reactions, transfusion-related

infections, and iron overload. Of the three, iron overload is the most frequent.

Iron overload has been associated with endocrine dysfunction⁶¹ and cardiac disease.⁶² Deferoxamine has been effective in treating iron overload by chelating and removing much of the excess iron from the body. Deferoxamine must be administered intravenously, however, and treatment requires at least 8 hours each day for a week. An oral iron chelator, deferasirox, was approved by the Food and Drug Administration in 2005.⁶³ Deferasirox is consumed in the morning as a slurry by dissolving several pills, but its effectiveness is yet to be determined.

Bone marrow or hematopoietic stem cell transplantation has proved successful for some individuals, but few patients qualify due to the lack of HLA-matched, related donors.⁶⁴ The event-free survival rates for patients receiving transplants from HLA-identical related donors are between 80% and 90% for SCD.⁶⁵⁻⁶⁸ Patients chosen for transplantation are generally children younger than age 17 with severe complications of SCD (i.e., stroke, acute chest syndrome, and refractory pain). In addition, morbidity and mortality following transplantation increase with age, which places another restriction on transplantation therapy.⁶⁹ There is evidence that transplantation restores some splenic function, but its effect on established organ damage is unknown.⁷⁰ Transplantation of cord blood stem cells from HLA-identical related and unrelated donors is associated with a disease-free survival rate of 90%.⁷¹ The primary benefit of using cord blood as a source of stem cells is that banking of cord blood increases the number of units available to achieve an HLA match.⁷¹ Some researchers are now focusing on the use of in utero stem cell transplantation to produce engraftment while the immune system of the fetus is prone to HLA tolerance. Others are attempting to genetically alter fetal hematopoietic stems cells to overcome HLA mismatches.⁷²

Hydroxycarbamide (hydroxyurea) therapy has offered some promise in relieving the sickling disorder by increasing the proportion of Hb F in the erythrocytes of individuals with SCD.⁷³ Hydroxyurea, given at 25 to 30 mg/kg, has been shown to reduce symptoms and prolong life, in part by increasing Hb F levels. Daily dosing produces a better HbF response compared to sequential weekly dosing.⁷⁴ Because Hb F does not copolymerize with Hb S, if the production of Hb F can be sufficiently augmented, the complications of SCD might be avoided. The severity of the disease expression and the number of irreversible sickle cells are inversely proportional to the extent to which Hb F synthesis persists. Individuals in whom Hb F levels stabilize at 12% to 20% of total hemoglobin may have little or no anemia and few, if any, vasoocclusive attacks. Levels of 4% to 5% Hb F may modulate the disease, and levels of 5% to 12% may suppress the severity of hemolysis and lessen the frequency of severe episodes.³⁴ Drug compliance is best monitored by an increasing MCV, while a decreasing LD might be an indicator of treatment response.³³ Response to hydroxycarbamide is variable among SCD patients, but high baseline Hb F level, neutrophil levels, and reticulocyte count are the best predictors of Hb F response.⁷⁵

Prevention of intracellular RBC dehydration reduces intracellular HbS polymerization thus reducing VOC. The uses of senicapoc to inhibit Gardos channels and Mg⁺⁺ to modulate K⁺-Cl⁻ transport systems show increased hemoglobin levels and decreased numbers of dense RBCs, resulting in reduced hemolysis but no clear reduction in VOC.⁷⁶⁻⁸⁰

Course and Prognosis

Proper management of SCD has increased the life expectancy of patients from 14 years in 1973 to the current average life span of 50 years.⁸¹ For men and women who are compound heterozygotes for Hb SC, the average life span is 60 and 68 years, respectively, with a few patients living into their seventies.^{30,82} Individuals with Hb SS can pursue a wide range of vocations and professions. They are discouraged, however, from jobs that require strenuous physical exertion or exposure to high altitudes or extreme environmental temperature variations.

Newborn screening for hemoglobinopathies has significantly reduced mortality in children with SCD by enabling prompt and comprehensive medical care. The most common form of screening is HPLC followed by confirmation using hemoglobin electrophoresis and genotyping methods.⁸³

Sickle Cell Trait

The term *sickle cell trait* refers to the heterozygous state (Hb AS) and describes a benign condition that generally does not affect mortality or morbidity except under conditions of extreme exertion. The trait occurs in approximately 8% of African Americans. It also can be found in Central Americans, Asians, and people from the region around the Mediterranean.¹

Individuals with sickle cell trait are generally asymptomatic and present with no significant clinical or hematologic manifestations. Under extremely hypoxic conditions, however, systemic sickling and vascular occlusion with pooling of sickled cells in the spleen, focal necrosis in the brain, rhabdomyolysis, and even death can occur. In circumstances such as severe respiratory infection, unpressurized flight at high altitudes, and anesthesia in which pH and oxygen levels are sufficiently lowered to cause sickling, patients may develop splenic infarcts.⁸ Failure to concentrate urine is the only consistent abnormality found in patients with sickle cell trait.⁸⁴ This abnormality is caused by diminished perfusion of the vasa recta of the kidney, which impairs concentration of urine by the renal tubules. Renal papillary necrosis with hematuria has been described in some patients.⁸

Although much controversy exists as to the potential connection between strenuous exercise and severe to fatal adverse events in patients with sickle cell trait, at least 46 cases have been documented in the literature (39 military recruits and 7 athletes).⁸⁵ The causes of these deaths were largely due to cardiac failures, renal failures, rhabdomyolysis, and heart illness. Opponents of the connection of sickle cell trait and fatal events argue that these events occur in sickle cell-negative people, many people with sickle cell trait do not develop adverse events, fatal sickle crisis cannot be adequately established in the patients encountering events, and

similar events have not been clearly documented in patients with sickle cell disease. However, it has been shown that military recruits with sickle cell trait have a 21 times greater risk of exercise-related death than recruits with normal hemoglobin.⁸⁵ Similar data have not been established in athletes with sickle cell trait.⁸⁵

The peripheral blood film of a patient with sickle cell trait shows normal RBC morphology, with the exception of a few target cells. No abnormalities in the leukocytes and thrombocytes are seen. The hemoglobin solubility screening test yields positive results, and sickle cell trait is diagnosed by detecting the presence of Hb S and Hb A on hemoglobin electrophoresis or HPLC. In individuals with sickle cell trait, electrophoresis reveals approximately 40% or less Hb S and approximately 60% or more Hb A, Hb A₂ level is normal or slightly increased, and Hb F level is within the reference interval. Levels of Hb S less than 40% can be seen in patients who also have α -thalassemia or iron or folate deficiency.²⁰ No treatment is required for this benign condition, and the patient's life span is not affected by sickle cell trait.

HEMOGLOBIN C

Hb C was the next hemoglobinopathy after Hb S to be described and in the United States is found almost exclusively in the African-American population. Spaet and Ranney reported this disease in the homozygous state (Hb CC) in 1953.⁸

Prevalence, Etiology, and Pathophysiology

Hb C is found in 17% to 28% of people of West African extraction and in 2% to 3% of African Americans.⁴ It is the most common nonsickling variant encountered in the United States and the third most common in the world.⁴ Hb C is defined by the structural formula $\alpha_2\beta_2^{6\text{Glu}\rightarrow\text{Lys}}$, in which lysine is substituted for glutamic acid in position 6 of the β chain. Lysine has a +1 charge and glutamic acid has a -1 charge, so the result of this substitution is a net change in charge of +2, which has a different structural effect on the hemoglobin molecule than the Hb S substitution.

Hb C is inherited in the same manner as Hb S but manifests as a milder disease. Similar to Hb S, Hb C polymerizes under low oxygen tension, but the structure of the polymers differs. Hb S polymers are long and thin, whereas the polymers in Hb C form a short, thick crystal within the RBCs. The shorter Hb C crystal does not alter RBC shape to the extent that Hb S does, so there is less splenic sequestration and hemolysis. In addition, vasoocclusive crisis does not occur.

Laboratory Diagnosis

A mild to moderate, normochromic, normocytic anemia occurs in homozygous Hb C disease. Occasionally, some microcytosis and mild hypochromia may be present. There is a marked increase in the number of target cells, a slight to moderate increase in the number of reticulocytes, and nucleated RBCs may be present in the peripheral blood.

Hexagonal crystals of Hb C form within the erythrocyte and may be seen on the peripheral blood film (Figure 27-10). Many

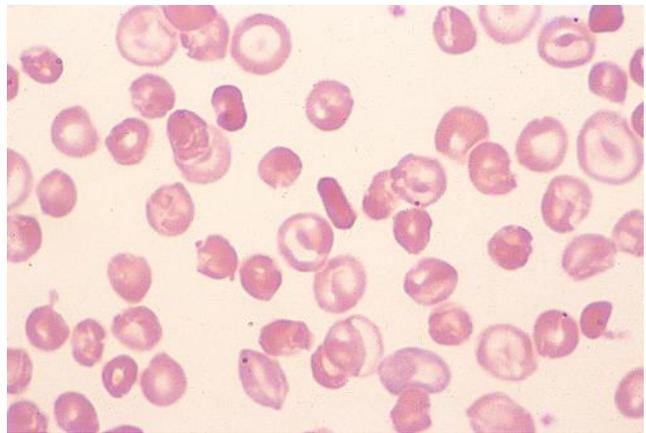


Figure 27-10 Peripheral blood film for a patient with hemoglobin C disease showing one Hb C crystal and target and folded cells ($\times 1000$). (Courtesy Ann Bell, University of Tennessee, Memphis.)

crystals appear extracellularly with no evidence of a cell membrane.^{86,87} In some cells, the hemoglobin is concentrated within the boundary of the crystal. The crystals are densely stained and vary in size and appear oblong with pyramid-shaped or pointed ends. These crystals may be seen on wet preparations by washing RBCs and resuspending them in a solution of sodium citrate.¹¹

Hb C yields a negative result on the hemoglobin solubility test, and definitive diagnosis is made using electrophoresis or HPLC. No Hb A is present in Hb CC disease. In addition, Hb C is present at levels of greater than 90%, with Hb F at less than 7% and Hb A₂ at approximately 2%. In Hb AC trait, about 60% Hb A and 30% Hb C are present. On cellulose acetate electrophoresis at an alkaline pH, Hb C migrates in the same position as Hb A₂, Hb E, and Hb O-Arab (Figure 27-7). Hb C is separated from these other hemoglobins on citrate agar electrophoresis at an acid pH (Figure 27-7). No specific treatment is required. This disorder becomes problematic only if infection occurs or if mild chronic hemolysis leads to gallbladder disease.

HEMOGLOBIN C-HARLEM (HEMOGLOBIN C-GEORGETOWN)

Hb C-Harlem (Hb C-Georgetown) has a double substitution on the β chain.^{5,20} The substitution of valine for glutamic acid at position 6 of the β chain is identical to the Hb S substitution, and the substitution at position 73 of aspartic acid for asparagine is the same as that in the Hb Korle Bu mutation. The double mutation is termed *Hb C-Harlem* (*Hb C-Georgetown*) because the abnormal hemoglobin migrates with Hb C on cellulose acetate electrophoresis at an alkaline pH. Patients heterozygous for this anomaly are asymptomatic, but patients with compound heterozygosity for Hb S and Hb C-Harlem have crises similar to those in Hb SS disease.⁸⁸

A positive solubility test result may occur with Hb C-Harlem, and hemoglobin electrophoresis or HPLC is necessary to confirm the diagnosis. On cellulose acetate at pH 8.4, Hb C-Harlem migrates in the C position (Figure 27-7). Citrate agar electrophoresis at pH 6.2, however, shows migration of Hb C-Harlem in the S position (Figure 27-7). Because so few cases have been

identified, the clinical outcome for homozygous individuals affected with this abnormality is uncertain,⁸⁸ but heterozygotes appear normal.

HEMOGLOBIN E

Prevalence, Etiology, and Pathophysiology

Hb E was first described in 1954.⁸⁹ The variant has a prevalence of 30% in Southeast Asia. As a result of the influx of immigrants from this area, Hb E prevalence has increased in the United States.⁹⁰ It occurs infrequently in African Americans and whites. Hb E is a β chain variant in which lysine is substituted for glutamic acid in position 26 ($\alpha_2\beta_2^{26\text{Glu}\rightarrow\text{Lys}}$). As with Hb C, this substitution results in a net change in charge of +2, but because of the position of the substitution, hemoglobin polymerization does not occur. However, the amino acid substitution at codon 26 inserts a cryptic splice site that causes abnormal alternative splicing and decreased transcription of functional mRNA for the Hb E globin chain.⁹¹ Thus the Hb E mutation is both a qualitative defect (due to the amino acid substitution in the globin chain) and a quantitative defect with a β -thalassemia phenotype (due to the decreased production of the globin chain).⁹¹

Clinical Features

The homozygous state (Hb EE) manifests as a mild anemia with microcytes and target cells. The RBC survival time is shortened. The condition is not associated with clinically observable icterus, hemolysis, or splenomegaly. The main concern in identifying homozygous Hb E is differentiating it from iron deficiency, β -thalassemia trait, and Hb E- β -thal (Chapter 28).⁹¹ The disease, Hb EE, resembles thalassemia trait. Because the highest incidence of the Hb E gene is in the areas of Thailand where malaria is most prevalent, it is thought that *P. falciparum* multiplies more slowly in Hb EE RBCs than in Hb AE or Hb AA RBCs and that the mutation may give some protection against malaria.¹ Hb E trait is asymptomatic. When Hb E is combined with β -thalassemia, however, the disease becomes more severe than Hb EE and more closely resembles β -thalassemia major, requiring regular blood transfusions.¹

Laboratory Diagnosis

Hb E does not produce a positive hemoglobin solubility test result and must be confirmed using electrophoresis or HPLC. In the homozygous state there is greater than 90% Hb E, a very low MCV (55 to 65 fL), few to many target cells, and a normal reticulocyte count. The heterozygous state has a mean MCV of 65 fL, slight erythrocytosis, target cells¹ (Figure 27-11), and approximately 30% to 40% Hb E. On cellulose acetate electrophoresis at an alkaline pH, Hb E migrates with Hb C, Hb O, and Hb A₂ (Figure 27-7). On citrate agar electrophoresis at an acid pH, Hb E can be separated from Hb C, but it comigrates with Hb A and Hb O (Figure 27-7).

Treatment and Prognosis

No therapy is required with Hb E disease and trait. Some patients may experience splenomegaly and fatigue, however.

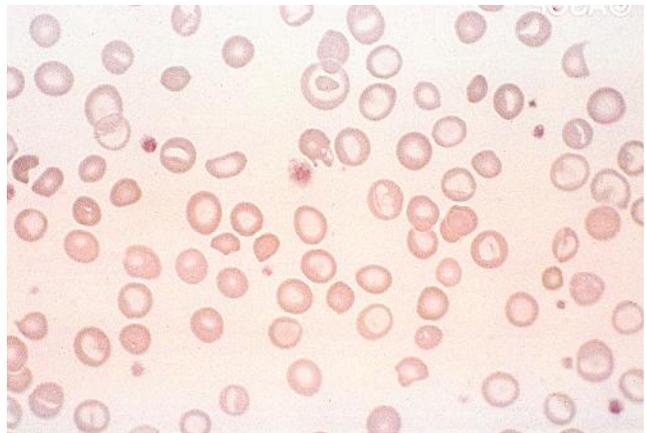


Figure 27-11 Microcytes and target cells in a patient with hemoglobin E trait. (From Hematology tech sample H-1, Chicago, 1991, American Society of Clinical Pathologists.)

Genetic counseling is recommended, and the Hb E gene mutation should be discussed in the same manner as a mild β -thalassemia allele.⁹¹

HEMOGLOBIN O-ARAB

Hb O-Arab is a β chain variant caused by the substitution of lysine for glutamic acid at amino acid position 121 ($\alpha_2\beta_2^{121\text{Glu}\rightarrow\text{Lys}}$).^{5,20,32} It is a rare disorder found in Kenya, Israel, Egypt, and Bulgaria and in 0.4% of African Americans. No clinical symptoms are exhibited by individuals who carry this variant, except for a mild splenomegaly in homozygotes.⁵ When Hb O-Arab is inherited with Hb S, however, severe clinical conditions similar to those in Hb SS result.⁵

Homozygous individuals have a mild hemolytic anemia, with many target cells on the peripheral blood film and a negative result on the hemoglobin solubility test. The presence of this hemoglobin variant must be confirmed using electrophoresis or HPLC. Because Hb O-Arab migrates with Hb A₂, Hb C, and Hb E on cellulose acetate at an alkaline pH, citrate agar electrophoresis at an acid pH is required to differentiate it from Hb C (Figure 27-7). Hb O-Arab is the only hemoglobin to move just slightly away from the point of application toward the cathode on citrate agar at an acid pH. No treatment is generally necessary for individuals with Hb O-Arab.

HEMOGLOBIN D AND HEMOGLOBIN G

Hb D and Hb G are a group of at least 16 β chain variants (Hb D) and 6 α chain variants (Hb G) that migrate in an alkaline pH at the same electrophoretic position as Hb S.^{4,8,20,92} This is because their α and β subunits have one fewer negative charge at an alkaline pH than Hb A, as does Hb S. They do not sickle, however, when exposed to reduced oxygen tension.

Most variants are named for the place where they were discovered. Hb D-Punjab and Hb D-Los Angeles are identical hemoglobins in which glutamine is substituted for glutamic acid at position 121 in the β chain ($\alpha_2\beta_2^{121\text{Glu}\rightarrow\text{Gln}}$). Hb D-Punjab

occurs in about 3% of the population in northwestern India, and Hb D-Los Angeles is seen in fewer than 2% of African Americans.

Hb G-Philadelphia is an α chain variant of the G hemoglobins, with a substitution of asparagine by lysine at position 68 ($\alpha_2^{68\text{Asn}\rightarrow\text{Lys}}\beta_2$).⁵ The Hb G-Philadelphia variant is the most common G variant encountered in African Americans and is seen with greater frequency than the Hb D variants. The Hb G variant is also found in Ghana.^{4,8,20,92}

Hb D and Hb G do not sickle and yield a negative hemoglobin solubility test result. On alkaline electrophoresis, Hb D and Hb G have the same mobility as Hb S (Figure 27-7). Hb D and Hb G can be separated from Hb S on citrate agar at pH 6.0 (Figure 27-7). These variants should be suspected whenever a hemoglobin is encountered that migrates in the S position on alkaline electrophoresis and has a negative result on the hemoglobin solubility test. In the homozygous state (Hb DD), there is greater than 95% Hb D, with normal amounts of Hb A₂ and Hb F.³⁰ Hb DD can be confused with the compound heterozygous state for Hb D and β^0 -thalassemia. The two disorders can be differentiated on the basis of the MCV, levels of Hb A₂, and family studies.^{4,8,20,92}

Hb D and Hb G are asymptomatic in the heterozygous state. Hb D disease (Hb DD) is marked by mild hemolytic anemia and chronic nonprogressive splenomegaly. No treatment is required.^{4,8,20,92}

COMPOUND HETEROZYGOSITY WITH HEMOGLOBIN S AND ANOTHER β -GLOBIN GENE MUTATION

Compound heterozygosity is the inheritance of two different mutant genes that share a common genetic locus—in this case the β -globin gene locus.⁹³⁻⁹⁶ Because there are two β -globin genes, these compound heterozygotes have inherited Hb S from one parent and another β chain hemoglobinopathy or thalassemia from the other parent. Compound heterozygosity of Hb S with Hb C, Hb D, Hb O, or β -thalassemia may produce hemolytic anemia of variable severity. Inheritance of Hb S with

other hemoglobins, such as Hb E, Hb G-Philadelphia, and Hb Korle Bu, causes disorders of no clinical consequence.⁹³

Hemoglobin SC

Hb SC is the most common compound heterozygous syndrome that results in a structural defect in the hemoglobin molecule in which different amino acid substitutions are found on each of two β -globin chains. At position 6, glutamic acid is replaced by valine (Hb S) on one β -globin chain and by lysine (Hb C) on the other β -globin chain. The frequency of Hb SC is 25% in West Africa. The incidence in the United States is approximately 1 in 833 births per year.^{93,97}

Clinical Features

Hb SC disease resembles a mild SCD. Growth and development are delayed compared with normal children. Unlike Hb SS, Hb SC usually does not produce significant symptoms until the teenage years. Hb SC disease may cause all the vasoocclusive complications of sickle cell anemia, but the episodes are less frequent, and damage is less disabling. Hemolytic anemia is moderate, and many patients exhibit moderate splenomegaly. Proliferative retinopathy is more common and more severe than in sickle cell anemia.⁹⁸ Respiratory tract infections with *S. pneumoniae* are common.⁸

Patients with Hb SC disease live longer than patients with Hb SS and have fewer painful episodes, but this disorder is associated with considerable morbidity and mortality, especially after age 30.⁹⁹ In the United States, the median life span for men is 60 years and for women 68 years.³⁰

Laboratory Diagnosis

The complete blood count shows a mild normocytic, normochromic anemia with many of the features associated with sickle cell anemia. The hemoglobin level is usually 11 to 13 g/dL, and the reticulocyte count is 3% to 5%. On the peripheral blood film, there are a few sickle cells, target cells, and intraerythrocytic crystalline structures. Crystalline aggregates of hemoglobin (SC crystals) form in some cells, where they protrude from the membrane (Figure 27-12).^{93,96} Hb SC crystals often appear as a

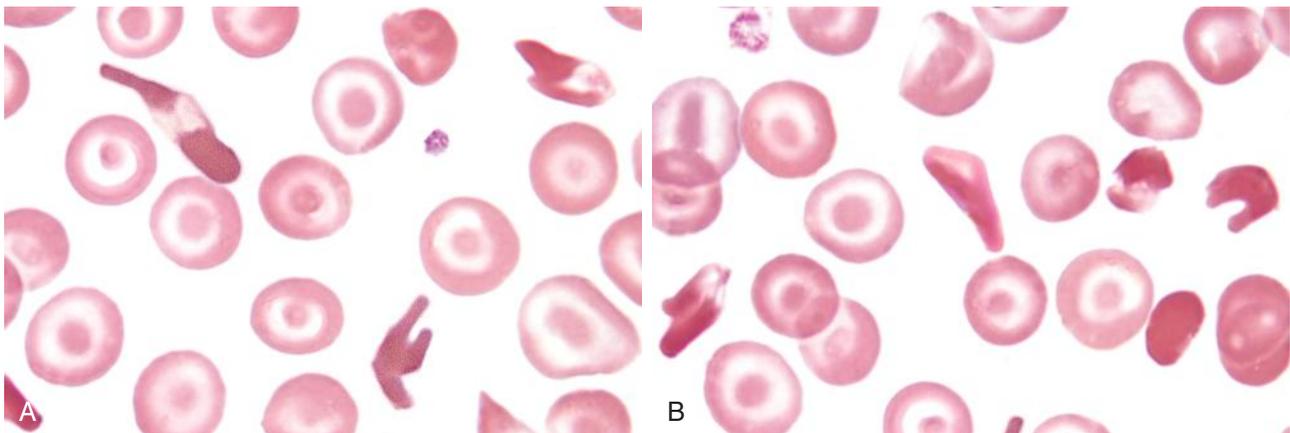


Figure 27-12 A and B, Peripheral blood film for a patient with hemoglobin SC. Note intraerythrocytic, blunt-ended SC crystals and target cells ($\times 1000$). (Courtesy Ann Bell, University of Tennessee, Memphis.)

hybrid of Hb S and Hb C crystals. They are longer than Hb C crystals but shorter and thicker than Hb S polymers and are often branched.

The result of the hemoglobin solubility screening test is positive because of the presence of Hb S. Electrophoretically, Hb C and Hb S migrate in almost equal amounts (45%) on cellulose acetate, and Hb F is normal. Hb C is confirmed on citrate agar at an acid pH, where it is separated from Hb E and Hb O. Hb A₂ migrates with Hb C, and its quantitation is of no consequence in Hb SC disease. Determination of Hb A₂ becomes vital, however, if a patient is suspected of having Hb C concurrent with β -thalassemia (Chapter 28).

Treatment and Prognosis

Therapy similar to that for SCD is given to individuals with Hb SC disease.⁸⁸

Hemoglobin S- β -Thalassemia

Compound heterozygosity for Hb S and β -thalassemia is the most common cause of sickle cell syndrome in patients of Mediterranean descent and is second to Hb SC disease among all compound heterozygous sickle disorders. Hb S- β -thal usually causes a clinical syndrome resembling that of mild or moderate sickle cell anemia. The severity of this compound heterozygous condition depends on the β chain production of the affected β -thalassemia gene. If there is no β -globin chain production from the β -thalassemia gene (Hb S- β^0 -thal), the clinical course is similar to that of homozygous sickle cell anemia. If there is production of a normal β -globin chain (Hb S- β^+ -thal), patients tend to have a milder condition than patients with Hb SC. These patients can be distinguished from individuals with sickle cell trait because of the presence of greater amounts of Hb S than of Hb A, increased levels of Hb A₂ and Hb F, microcytosis from the thalassemia, hemolytic anemia, abnormal peripheral blood morphology, and splenomegaly (Chapter 28).^{20,93}

Hemoglobin SD and Hemoglobin SG-Philadelphia

Hb SD is a compound heterozygous and Hb SG-Philadelphia a double heterozygous sickle cell syndrome.^{20,92} Hb SG-Philadelphia is asymptomatic because Hb G is associated with an α gene mutation that still allows for sufficient Hb A to be produced. Hb SD syndrome may cause a mild to severe hemolytic anemia because both β chains are affected. Some patients with Hb SD may have severe vasoocclusive complications. The Hb D syndrome in African Americans is usually due to the interaction of Hb S with Hb D-Los Angeles (Hb D-Punjab).

The peripheral blood film findings for Hb SD disease are comparable to those seen in less severe forms of Hb SS disease. Because Hb D and Hb G comigrate with Hb S on cellulose acetate electrophoresis at an alkaline pH, citrate agar electrophoresis at an acid pH is necessary to separate Hb S from Hb D and Hb G. The clinical picture is valuable in differentiating Hb SD and Hb SG. The treatment for Hb SD disease is similar to that

for patients with SCD and is administered according to the severity of the clinical condition.

Hemoglobin S/O-Arab and HbS/D-Punjab

Hb S/O-Arab and Hb S/D-Punjab are rare compound heterozygous hemoglobinopathies that cause severe chronic hemolytic anemia with vasoocclusive episodes.^{8,20,92} Both mutations replace glutamic acid at position 121; O-Arab substitutes lysine and D-Punjab substitutes glutamine. Glutamic acid at position 121 is located on the outer surface of the hemoglobin tetramer, which enhances the polymerization process involving Hb S. Hb S/O-Arab can be mistaken for Hb SC on cellulose acetate electrophoresis at an alkaline pH because Hb C and Hb O-Arab migrate at the same position; however, differentiation is easily made on citrate agar at an acid pH. Therapy for these patients is similar to that for patients with SCD. Similarly, Hb D-Punjab comigrates with Hb S on alkaline electrophoresis, making this mutation look like SCD. Hb O-Arab and Hb D-Punjab are not clinically significant in either the heterozygous or the homozygous form.¹

Hemoglobin S-Korle Bu

Hb Korle Bu is a rare hemoglobin variant with substitution of aspartic acid for asparagine at position 73 of the β chain.²⁰ When inherited with Hb S, it interferes with lateral contact between Hb S fibers by disrupting the hydrophobic pocket for β_6 valine, which inhibits Hb S polymerization. The compound heterozygous condition Hb S-Korle Bu is asymptomatic.

CONCOMITANT CIS MUTATIONS WITH HEMOGLOBIN S

A concomitant cis mutation with Hb S involves a second mutation on the same gene along with Hb S. Three cis mutations will be described: Hb C-Harlem, Hb S-Antilles, and Hb S-Oman.

Hemoglobin C-Harlem

Hb C-Harlem has two substitutions on the β chain: the sickle mutation and the Korle Bu mutation. Patients heterozygous for only Hb C-Harlem are asymptomatic. The compound heterozygous Hb S-Hb C-Harlem state resembles Hb SS clinically. Hb C-Harlem yields a positive result on the hemoglobin solubility test and migrates to the Hb C position on cellulose acetate electrophoresis at an alkaline pH and to the Hb S position on citrate agar electrophoresis at an acid pH.

Hemoglobin S-Antilles and Hemoglobin S-Oman

Hb S-Antilles bears the Hb S mutation ($\beta^{6\text{Glu}\rightarrow\text{Val}}$) along with a substitution of isoleucine for valine at position 23.¹⁰⁰ Hb S-Oman also has the Hb S mutation with a second substitution of lysine for glutamic acid at position 121.¹⁰¹ In both of these hemoglobin variants, the second mutation enhances Hb S such that significant sickling can occur even in heterozygotes.¹

Table 27-2 summarizes common clinically significant hemoglobinopathies, including general characteristics and treatment options.

TABLE 27-2 Common Clinically Significant Hemoglobinopathies

Hemoglobin Disorder	Abnormal Hemoglobin	Structural Defect	Groups Primarily Affected	Hemoglobin Solubility Test Results	Hemoglobins Present	Red Blood Cell Morphology	Symptoms/Organ Defects	Treatment
Sickle cell anemia (homozygous)	Hb S	$\alpha_2\beta_2^{6\text{Glu}\rightarrow\text{Val}}$	African, African American, Middle Eastern, Indian, Mediterranean	Positive	0% Hb A, >80% Hb S, 1%—20% Hb F, 2%—5% Hb A ₂	Sickle cells, target cells, nucleated RBCs, polychromasia, Howell-Jolly bodies, basophilic stippling	Vasocclusion, bacterial infections, hemolytic anemia, aplastic episodes; bones, lungs, liver, spleen, penis, eyes, central nervous system, urinary tract	Transfusions, antibiotics, analgesics, bone marrow transplant, hydroxyurea
Hb C disease (homozygous)	Hb C	$\alpha_2\beta_2^{6\text{Glu}\rightarrow\text{Lys}}$	African, African American	Negative	0% Hb A, >90% Hb C, <7% Hb F, 2% Hb A ₂	Hb C crystals, target cells, nucleated RBCs, occasionally some microcytes	Mild splenomegaly, mild hemolysis	Usually none, antibiotics
Hb SC-Harlem* (Hb C-George-town)	Hb C-Harlem, Hb S	$\alpha_2\beta_2^{6\text{Glu}\rightarrow\text{Val}}$ and $\alpha_2\beta_2^{73\text{Asp}\rightarrow\text{Asn}}$ on same gene and $\alpha_2\beta_2^{6\text{Glu}\rightarrow\text{Val}}$	Rare, so uncertain; African, African American	Positive	Hb C-Harlem migrates with Hb C at alkaline pH; migrates with Hb S at acid pH	Target cells	Compound heterozygotes with Hb SC-Harlem have symptoms similar to Hb SS	Similar to Hb SS
Hb E disease (homozygous)	Hb E	$\alpha_2\beta_2^{26\text{Glu}\rightarrow\text{Lys}}$	Southeast Asian, African, African American	Negative	0% Hb A, 95% Hb E, 2%–4% Hb A ₂ ; migrates with Hb A ₂ , Hb C, and Hb O at alkaline pH	Target cells, microcytes	Mild anemia, mild splenomegaly, no symptoms	Usually none
Hb O-Arab (homozygous)	Hb O-Arab	$\alpha_2\beta_2^{121\text{Glu}\rightarrow\text{Lys}}$	Kenyan, Israeli, Egyptian, Bulgarian, African American	Negative	0% Hb A, 95% Hb O, 2%–4% Hb A ₂ ; migrates with Hb A ₂ , Hb C, and Hb E at alkaline pH	Target cells	Mild splenomegaly	Usually none
Hb D disease (rare homozygous)	Hb D-Punjab (Hb-D Los Angeles)	$\alpha_2\beta_2^{121\text{Glu}\rightarrow\text{Gln}}$	Middle Eastern, Indian	Negative	95% Hb D, normal Hb A ₂ and Hb F; migrates with Hb S at alkaline pH	Target cells	Mild hemolytic anemia, mild splenomegaly	Usually none

Continued

TABLE 27-2 Common Clinically Significant Hemoglobinopathies—cont'd

Hemoglobin Disorder	Abnormal Hemoglobin	Structural Defect	Groups Primarily Affected	Hemoglobin Solubility Test Results	Hemoglobins Present	Red Blood Cell Morphology	Symptoms/Organ Defects	Treatment
Hb G disease (rare homozygous)	Hb G, Hb G-Philadelphia	$\alpha_2\beta_2^{68Asn\rightarrow Lys}$	African American, Ghanaian	Negative	95% Hb G, normal Hb A ₂ and Hb F; migrates with S at alkaline pH	Target cells	Mild hemolytic anemia, mild splenomegaly	Usually none
Hb SC* disease	Hb S, Hb C	$\alpha_2\beta_2^{6Glu\rightarrow Val}$ and $\alpha_2\beta_2^{6Glu\rightarrow Lys}$	Same as Hb S	Positive	45% Hb S, 45% Hb C, 2%–4% Hb A ₂ , 1% Hb F	Sickle cells, Hb SC crystals, target cells	Same as those for Hb SS except milder	Similar to that for Hb SS but less intensive
Hb S- β -thalassemia*	Hb S + β -thalassemia mutation	$\alpha_2\beta_2^{6Glu\rightarrow Val}$ and β^0 or β^+	Same as Hb S	Positive	Hb S variable, some Hb A in β^+ , increased Hb A ₂ and Hb F	Sickle cells, target cells, microcytes	Hemolytic anemia, splenomegaly	Similar to that for Hb SS; varies depending on amount of Hb A present
Hb SD* disease	Hb S, Hb D	$\alpha_2\beta_2^{6Glu\rightarrow Val}$ and $\alpha_2\beta_2^{121Glu\rightarrow Gln}$	Same as Hb S	Positive	45% Hb S, 45% Hb D, 2%–4% Hb A ₂ , 1% Hb F; Hb S and D comigrate at alkaline pH	Sickle cells, target cells	Similar to those for Hb SS but milder	Similar to that for Hb SS but less intensive
Hb SG†	Hb S, Hb G	$\alpha_2\beta_2^{6Glu\rightarrow Val}$ and $\alpha_2\beta_2^{68Asn\rightarrow Lys}$	Same as Hb S	Positive	45% Hb S, 45% Hb G, 2%–4% Hb A ₂ , 1% Hb F; Hb S and G comigrate at alkaline pH	Target cells	No symptoms	Usually none
Hb SO-Arab*	Hb S, Hb O-Arab	$\alpha_2\beta_2^{6Glu\rightarrow Val}$ and $\alpha_2\beta_2^{121Glu\rightarrow Lys}$	Same as Hb S	Positive	45% Hb S, 45% Hb O, 2%–4% Hb A ₂ , 1% Hb F	Sickle cells, target cells	Similar to those for Hb SS	Similar to that for Hb SS

*Compound heterozygous.

†Double heterozygous.

Asn, Asparagine; Asp, aspartic acid; Gln, glutamine; Glu, glutamic acid; Hb, hemoglobin; Lys, lysine; Val, valine.

HEMOGLOBIN M

Hb M is caused by a variety of mutations in the α -, β -, and γ -globin genes, all of which result in the production of methemoglobin—hence the Hb M designation.^{92,102} These genetic mutations result in a structural abnormality in the globin portion of the molecule. Most M hemoglobins involve a substitution of a tyrosine amino acid for either the proximal (F₈) or the distal (E₇) histidine amino acid in the α , β , or γ chains. These substitutions cause heme iron to auto-oxidize, which results in methemoglobinemia. Hb M has iron in the ferric state (Fe³⁺) and is unable to carry oxygen, which produces cyanosis. Seven hemoglobin variants affecting the α or β chains have been classified as M hemoglobins: Hb M-Boston, Hb M-Iwate, and Hb Auckland (α chain variants); and Hb Chile, Hb M-Saskatoon, Hb M-Milwaukee-1, and Hb M-Milwaukee-2 (β chain variants), all named for the locations in which they were discovered.⁵ Two variants affect the γ chain—Hb F-M-Osaka and Hb F-M-Fort Ripley⁵—but symptoms disappear when Hb A replaces Hb F at 3 to 6 months of age.

Hb M variants have altered oxygen affinity and are inherited as autosomal dominant disorders. Affected individuals have 30% to 50% methemoglobin (healthy individuals have less than 1%) and may appear cyanotic. Ingestion of oxidant drugs, such as sulfonamides, can increase methemoglobin to life-threatening levels. Methemoglobin causes the blood specimen to appear brown. Heinz bodies may be seen sometimes on wet preparations because methemoglobin causes globin chains to precipitate (see Figure 14-11). Diagnosis is made by spectral absorption of the hemolysate or by hemoglobin electrophoresis. The absorption spectrum peaks are determined at various wavelengths. The unique absorption range of each Hb M variant is identified when these are compared with the spectrum of normal blood.

Before electrophoresis, all hemoglobin types are converted to methemoglobin by adding potassium cyanide to the sample so that any migration differences observed are only due to an amino acid substitution, not differences in iron states. On cellulose acetate, Hb M migrates slightly more slowly than Hb A. The electrophoresis should be performed on agar gel at pH 7.1 for clear separation. Further confirmation may be obtained using HPLC or deoxyribonucleic acid (DNA)-based globin gene analysis. No treatment is necessary. Diagnosis is essential to prevent inappropriate treatment for other conditions, such as cyanotic heart disease.

UNSTABLE HEMOGLOBIN VARIANTS

Unstable hemoglobin variants result from genetic mutations to globin genes creating hemoglobin products that precipitate in vivo, producing Heinz bodies and causing a hemolytic anemia.^{92,102} More than 140 variants of unstable hemoglobin exist.⁵ The majority of these are β chain variants, and most others are α chain variants. Only a few are γ and δ chain variants. Most unstable hemoglobin variants have no clinical significance, although the majority has an increased oxygen affinity. About 25% of unstable hemoglobins are responsible for hemolytic

anemia, which varies from compensated mild anemia to severe hemolytic episodes.

At one time, the anemia was referred to as *congenital nonspherocytic hemolytic anemia* or *congenital Heinz body anemia*. This disorder is more properly called *unstable hemoglobin disease*. The syndrome appears at or just after birth, depending on the globin chains involved. It is inherited in an autosomal dominant pattern. All patients are heterozygous; apparently the homozygous condition is incompatible with life. The instability of the hemoglobin molecule may be due to (1) substitution of a charged for an uncharged amino acid in the interior of the molecule, (2) substitution of a polar for a nonpolar amino acid in the hydrophobic heme pocket, (3) substitution of an amino acid in the α and β chains at the intersubunit contact points, (4) replacement of an amino acid with proline in the α helix section of a chain, and (5) deletion or elongation of the primary structure.

Clinical Features

The unstable hemoglobin disorder is usually detected in early childhood in patients with hemolytic anemia accompanied by jaundice and splenomegaly. Fever or ingestion of an oxidant exacerbates the hemolysis. The severity of the anemia depends on the degree of instability of the hemoglobin molecule. The unstable hemoglobin precipitates in vivo and in vitro in response to factors that do not affect normal hemoglobins, such as drug ingestion and exposure to heat or cold. The hemoglobin precipitates in the RBC as Heinz bodies. The precipitated hemoglobin attaches to the cell membrane, causing clustering of band 3, attachment of autologous immunoglobulin, and macrophage activation. In addition, Heinz bodies can be trapped mechanically in the splenic sieve, which shortens RBC survival. The oxygen affinity of these cells is also abnormal.

The most prevalent unstable hemoglobin is Hb Köln. Other unstable hemoglobins include Hb Hammersmith, Hb Zurich, Hb Gun Hill, and Hb Hammersmith.⁵ Because of the large variability in the degree of instability in these hemoglobins, the extent of hemolysis varies greatly. For some of the variants, such as Hb Zurich, the presence of an oxidant is required for any significant hemolysis to occur.

Laboratory Diagnosis

The RBC morphology varies. It may be normal or show slight hypochromia and prominent basophilic stippling, which possibly is caused by excessive clumping of ribosomes. Before splenectomy, the hemoglobin level ranges from 7 to 12 g/dL, with a 4% to 20% reticulocyte count. After splenectomy, anemia is corrected, but reticulocytosis persists. Heinz bodies can be shown using a supravital stain (see Figure 14-11). After splenectomy, Heinz bodies are larger and more numerous. Many patients excrete dark urine that contains dipyrrole.

Many unstable hemoglobins migrate in the normal AA pattern and thus are not detected on electrophoresis. Other tests used to detect unstable hemoglobins include the isopropanol precipitation test, which is based on the principle that an isopropanol solution at 37° C weakens the bonding forces of the

hemoglobin molecule. If unstable hemoglobins are present, rapid precipitation occurs in 5 minutes, and heavy flocculation occurs after 20 minutes. Normal hemoglobin does not begin to precipitate until after approximately 40 minutes. The heat denaturation test also can be used. When incubated at 50° C for 1 hour, heat-sensitive unstable hemoglobins show a flocculent precipitation, whereas normal hemoglobin shows little or no precipitation. Significant numbers of Heinz bodies appear after splenectomy, but even in individuals with intact spleens, with longer incubation and the addition of an oxidative substance such as acetylphenylhydrazine, unstable hemoglobins form more Heinz bodies than does the blood from individuals with normal hemoglobins. Other techniques, such as isoelectric focusing, can resolve many hemoglobin variants with only a slight alteration in their isoelectric point, and globin chain analysis can be performed by HPLC or DNA-based globin gene analysis.

Treatment and Prognosis

Patients are treated to prevent hemolytic crises. In severe cases, the spleen must be removed to reduce sequestration and rate of removal of RBCs. Because unstable hemoglobin disease is rare, prognosis in the affected individuals is unclear. Patients are cautioned against the use of sulfonamides and other oxidant drugs. They also should be informed of the potential for febrile illnesses to trigger a hemolytic episode.

HEMOGLOBINS WITH INCREASED AND DECREASED OXYGEN AFFINITY

More than 150 hemoglobin variants have been discovered to have abnormal oxygen affinity.^{4,102-104} Most are high-affinity variants and have been associated with familial erythrocytosis. The remaining hemoglobin variants are characterized by low oxygen affinity. Many of these are associated with mild to moderate anemia.²

As described in Chapter 10, normal Hb A undergoes a series of allosteric conformational changes as it converts from a fully deoxygenated to a fully oxygenated form. These conformational changes affect hemoglobin function and its affinity for oxygen. When normal hemoglobin is fully deoxygenated (tense state), it has low affinity for oxygen and other heme ligands and high affinity for allosteric effectors, such as Bohr protons and 2,3-bisphosphoglycerate. In the oxygenated (relaxed) state, hemoglobin has a high affinity for heme ligands, such as oxygen, and a low affinity for Bohr protons and 2,3-bisphosphoglycerate. The transition from the tense to the relaxed state involves a series of structural changes that have a marked effect on hemoglobin function. If an amino acid substitution lowers the stability of the tense structure, the transition to the relaxed state occurs at an earlier stage in ligand binding, and the hemoglobin has increased oxygen affinity and decreased heme-heme interaction or cooperativity (Chapter 10). One example of a β chain variant is Hb Kempsey. This unstable hemoglobin variant has amino acid substitutions at sites crucial to hemoglobin function.

Hemoglobins with Increased Oxygen Affinity

The high-affinity variants, like other structurally abnormal hemoglobins, show an autosomal dominant pattern of inheritance. Affected individuals have equal volumes of Hb A and the abnormal variant. Exceptions to this are compound heterozygotes for Hb Abruazzo and β -thalassemia and for Hb Crete and β -thalassemia, in which the proportion of abnormal hemoglobin is greater than 85%.

More than 90 variant hemoglobins with high oxygen affinity have been discovered. Such hemoglobins fail to release oxygen on demand, and hypoxia results. The kidneys sense the hypoxia and respond by increasing the release of erythropoietin, which leads to a compensatory erythrocytosis. These variants differ from unstable hemoglobin, which also may have abnormal oxygen affinity, in that they do not precipitate *in vivo* to produce hemolysis and there is no abnormal RBC morphology.

Most individuals are asymptomatic and show no physical symptoms except a ruddy complexion. Erythrocytosis is usually detected during routine examination because the patient generally has a high RBC count, hemoglobin, and hematocrit. The WBC count, platelet count, and peripheral blood film findings are generally normal. In some cases, hemoglobin electrophoresis may establish a diagnosis. An abnormal band that separates from the A band is present on cellulose acetate in some variants; however, if a band is not found, the diagnosis of increased oxygen affinity cannot be ruled out. In some cases the abnormal hemoglobin can be separated by using citrate agar (pH 6.0) or by gel electrophoresis. Measurement of oxygen affinity is required for definitive diagnosis.

Patients with high-oxygen-affinity hemoglobins live normal lives and require no treatment. Diagnosis should be made to avoid unnecessary treatment of the erythrocytosis as a myeloproliferative neoplasm or a secondary erythrocytosis.

Hemoglobins with Decreased Oxygen Affinity

Hemoglobins with decreased oxygen affinity quickly release oxygen to the tissues, which results in normal to decreased hemoglobin concentration and slight anemia. The best known of these hemoglobins is Hb Kansas, which has an amino acid substitution of asparagine by threonine at position 102 of the β chain. These hemoglobins may be present when cyanosis and a normal arterial oxygen tension coexist, and most may be detected by starch gel electrophoresis, HPLC, or DNA-based globin gene analysis.

GLOBAL BURDEN OF HEMOGLOBINOPATHIES

The prevalence of hemoglobinopathies has already been presented in this chapter, and the bulk of these conditions occurs in underdeveloped countries. However, as developing countries work to decrease deaths from malnutrition, infectious diseases, and other conditions, more patients with hemoglobinopathies will survive and remain consumers of the health care system. For example, in 1944 thalassemia was first identified in Cypress. However, during the post-World War II

recovery period, as the death rate decreased, the prevalence of thalassemias increased.² In 1970 it was estimated that in the absence of systems to control the disease, within 40 years 78,000 units of blood would be needed each year, requiring that 40% of the population serve as donors.² If left unchecked, the cost to maintain thalassemia therapy would exceed the country's total health care budget. In contrast, efforts to de-

velop prenatal screening and genetic counseling programs have reduced the birth rate of SCD.² It is clear that hemoglobinopathies are a worldwide problem requiring planning, investment, and interventions from around the globe to optimize the impact on patients with the disease without debilitating the health care systems of developing countries where the disease is prevalent.

SUMMARY

- Hemoglobinopathies are genetic disorders of globin genes that produce structurally abnormal hemoglobins with altered amino acid sequences, which affect hemoglobin function and stability.
- Hb S is the most common hemoglobinopathy, resulting from a substitution of valine for glutamic acid at position 6 of the β globin chain, and primarily affects people of African descent.
- Hb S polymerizes in the RBCs because of abnormal interaction with adjacent tetramers when it is in the deoxygenated form, producing sickle-shaped RBCs.
- In homozygous Hb SS, the polymerization of hemoglobin may result in severe episodic conditions; however, factors other than hemoglobin polymerization may account for vasoocclusive episodes in sickle cell patients.
- The most clinically significant hemoglobinopathies are Hb SS, Hb SC, and Hb S- β -thalassemia; Hb SS causes the most severe disease.
- Individuals with sickle cell trait (Hb AS) are clinically asymptomatic.
- Sickle cell anemia (Hb SS) is a normocytic, normochromic anemia, characterized by a single band in the S position on hemoglobin electrophoresis, a single Hb S peak on HPLC, and a positive hemoglobin solubility test.
- The median life expectancy of patients with SCD has been extended to approximately 50 years.
- Hb C and Hb E are the next most common hemoglobinopathies after Hb S and cause mild hemolysis in the homozygous state. In the heterozygous states, these hemoglobinopathies are asymptomatic.
- Hb C is found primarily in people of African descent.
- On peripheral blood films from patients with Hb CC, hexagonal crystals may be seen with and without apparent RBC membrane surrounding them.
- Hb EE results in a microcytic anemia and is found primarily in people of Southeast Asian descent.
- Other variants, such as unstable hemoglobins and hemoglobins with altered oxygen affinity, can be identified, and many cause no clinical abnormality.
- Laboratory procedures employed for diagnosis of hemoglobinopathies are the CBC, peripheral blood film evaluation, reticulocyte count, hemoglobin solubility test, and methods to quantitate normal hemoglobins and variants including hemoglobin electrophoresis (acid and alkaline pH), high-performance liquid chromatography, and capillary electrophoresis.
- Advanced techniques available for hemoglobin identification include isoelectric focusing and DNA-based analysis of the globin genes.

Now that you have completed this chapter, go back and read again the case study at the beginning and respond to the questions presented.

REVIEW QUESTIONS

Answers can be found in the Appendix.

1. A qualitative abnormality in hemoglobin may involve all of the following *except*:
 - a. Replacement of one or more amino acids in a globin chain
 - b. Addition of one or more amino acids in a globin chain
 - c. Deletion of one or more amino acids in a globin chain
 - d. Decreased production of a globin chain
2. The substitution of valine for glutamic acid at position 6 of the β chain of hemoglobin results in hemoglobin that:
 - a. Is unstable and precipitates as Heinz bodies
 - b. Polymerizes to form tactoid crystals
 - c. Crystallizes in a hexagonal shape
 - d. Contains iron in the ferric (Fe^{3+}) state
3. Patients with SCD usually do not exhibit symptoms until 6 months of age because:
 - a. The mother's blood has a protective effect
 - b. Hemoglobin levels are higher in infants at birth
 - c. Higher levels of Hb F are present
 - d. The immune system is not fully developed
4. Megaloblastic episodes in SCD can be prevented by prophylactic administration of:
 - a. Iron
 - b. Folic acid
 - c. Steroids
 - d. Erythropoietin

5. Which of the following is the most definitive test for Hb S?
 - a. Hemoglobin solubility test
 - b. Hemoglobin electrophoresis at alkaline pH
 - c. Osmotic fragility test
 - d. Hemoglobin electrophoresis at acid pH
6. A patient presents with mild normochromic, normocytic anemia. On the peripheral blood film, there are a few target cells, rare nucleated RBCs, and hexagonal crystals within and lying outside of the RBCs. Which abnormality in the hemoglobin molecule is most likely?
 - a. Decreased production of β chains
 - b. Substitution of lysine for glutamic acid at position 6 of the β chain
 - c. Substitution of tyrosine for the proximal histidine in the β chain
 - d. Double amino acid substitution in the β chain
7. A well-mixed specimen obtained for a CBC has a brown color. The patient is being treated with a sulfonamide for a bladder infection. Which of the following could explain the brown color?
 - a. The patient has Hb M.
 - b. The patient is a compound heterozygote for Hb S and thalassemia.
 - c. The incorrect anticoagulant was used.
 - d. Levels of Hb F are high.
8. Through routine screening, prospective parents discover that they are both heterozygous for Hb S. What percentage of their children potentially could have sickle cell anemia (Hb SS)?
 - a. 0%
 - b. 25%
 - c. 50%
 - d. 100%
9. Painful crises in patients with SCD occur as a result of:
 - a. Splenic sequestration
 - b. Aplasia
 - c. Vasooclusion
 - d. Anemia
10. The screening test for Hb S that uses a reducing agent, such as sodium dithionite, is based on the fact that hemoglobins that sickle:
 - a. Are insoluble in reduced, deoxygenated form
 - b. Form methemoglobin more readily and cause a color change
 - c. Are unstable and precipitate as Heinz bodies
 - d. Oxidize quickly and cause turbidity
11. DNA analysis documents a patient has inherited the sickle mutation in both β -globin genes. The two terms that best describe this genotype are:
 - a. Homozygous/trait
 - b. Homozygous/disease
 - c. Heterozygous/trait
 - d. Heterozygous/disease
12. In which of the following geographic areas is Hb S most prevalent?
 - a. India
 - b. South Africa
 - c. United States
 - d. Sub-Saharan Africa
13. Which hemoglobinopathy is more common in Southeast Asian patients?
 - a. Hb S
 - b. Hb C
 - c. Hb O
 - d. Hb E
14. Which of the following Hb S compound heterozygote exhibits the mildest symptoms?
 - a. Hb S- β -Thal
 - b. Hb SG
 - c. Hb S-C-Harlem
 - d. Hb SC
15. A 1-year-old Indian patient presents with anemia, and both parents claim to have an "inherited anemia" but can't remember the type. The peripheral blood shows target cells, and the hemoglobin solubility is negative. Alkaline hemoglobin electrophoresis shows a single band at the "Hb C" position and a small band at the "Hb F" position. Acid hemoglobin electrophoresis shows two bands. The most likely diagnosis is:
 - a. Hb CC
 - b. Hb AC
 - c. Hb CO
 - d. Hb SC
16. Unstable hemoglobins show all of the following findings EXCEPT:
 - a. Globin chains precipitate intracellularly
 - b. Heinz body formation
 - c. Elevated reticulocyte count
 - d. Only homozygotes are symptomatic

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28

Thalassemias

Elaine M. Keohane*

OUTLINE

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Τηλασσεμια

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OBJECTIVES

After completion of this chapter, the reader will be able to:

1. Describe the hemoglobin defect found in thalassemias.
2. Discuss the geographic distribution of thalassemia and its association with malaria.
3. Name the chromosomes that contain the α -globin gene and the β -globin gene clusters and the globin chains produced by each.
4. Describe the type of genetic mutations that result in α - and β -thalassemias.
5. Explain the pathophysiologic effects caused by the imbalance of globin chain synthesis in α - and β -thalassemias.
6. Describe the four major clinical syndromes of β -thalassemia and the clinical expression of each heterozygous and homozygous form.
7. Recognize the pattern of laboratory findings in heterozygous and homozygous β -thalassemias, including hereditary persistence of fetal hemoglobin (HPFH).
8. Describe the treatment of homozygous β -thalassemias, the risks involved, and the reason it is necessary to monitor iron levels.
9. Correlate the clinical syndromes of α -thalassemia with the number of α genes present.
10. Recognize the laboratory findings associated with various α -thalassemia syndromes.
11. Describe the clinical syndromes of thalassemia associated with common structural hemoglobin variants.
12. Specify tests that are used for screening for β -thalassemia carriers.
13. Discuss the role of the complete blood count, peripheral blood film review, supravital stain, hemoglobin fraction quantification (using hemoglobin electrophoresis, high-performance liquid chromatography, and/or capillary zone electrophoresis), and molecular genetic testing in diagnosis of thalassemia syndromes.
14. Differentiate β -thalassemia minor from iron deficiency anemia.

CASE STUDY

After studying the material in this chapter, the reader should be able to respond to the following case study:

A 24-year-old male medical student in the United States was found to have a hemoglobin level of 10.2 g/dL in a hematology laboratory class. During discussion of the family history with this student, a hematologist at the university discovered that his mother had always been anemic, had periodically been given iron therapy, and had a history of several acute episodes of gallbladder disease (attacks). Both of the student's parents had been born in Sicily. A cousin on his mother's side had two children who died of thalassemia major at the ages of 4 and 5 years and had a third young daughter with thalassemia major who was being treated with regular blood transfusions. The student's laboratory test results were as follows:

Continued

* The author extends appreciation to Martha Payne, who provided the foundation of this chapter, and Rakesh P. Mehta, who authored this chapter in previous editions.

CASE STUDY—cont'd

	Patient Results	Reference Interval
RBC ($\times 10^{12}/L$)	5.74	4.60–6.00
HGB (g/dL)	10.2	14.0–18.0
HCT (%)	35	40–54
MCV (fL)	61.0	80–100
MCH (pg)	17.8	26–32
MCHC (g/dL)	29.1	32–36

Peripheral blood RBCs exhibited moderate microcytosis, slight hypochromia, and slight poikilocytosis with occasional target cells, and several RBCs had basophilic stippling. Hb A₂ was 4.9% of total hemoglobin by high-performance liquid chromatography (reference interval, 0% to 3.5%). Serum ferritin level was 320 ng/mL (reference interval, 15 to 400 ng/mL).

1. Why was the family history so important in this case, and what diagnosis did it suggest?

2. What laboratory values helped confirm the diagnosis?
3. From what other disorders should this anemia be differentiated? What laboratory tests would be helpful? Why is differentiation important?
4. If this individual was planning to have children, what genetic counseling should be done?

DEFINITIONS AND HISTORY

The thalassemias are a diverse group of inherited disorders caused by genetic mutations that reduce or prevent the synthesis of one or more of the globin chains of the hemoglobin (Hb) tetramer. In 1925, Cooley and Lee first described four children with anemia, splenomegaly, mild hepatomegaly, and mongoloid facies.¹ These characteristics would later become typical findings in young children with untreated β -thalassemia major, often referred to as *Cooley's anemia*. Seven years later, Whipple and Bradford published a paper outlining the detailed autopsy studies of children who died of this disorder.² Because of the high incidence of patients of Mediterranean descent with this disorder, Whipple called the disease *Thalassia* (Greek for “great sea”) anemia, which was subsequently changed to *thalassemia*.² Several investigators in the 1940s demonstrated the genetic basis for this anemia and were able to show that in patients who were homozygous for this condition (thalassemia major), the disease had a severe course. The heterozygotes, however, not only were carriers but also had a milder anemia (thalassemia minor). In the 1950s, thalassemias resulting from defects in the α -globin chain were described.²

Thalassemia results from a reduced or absent synthesis of one or more of the globin chains of hemoglobin. A wide variety of mutations in hemoglobin genes lead to clinical outcomes that are extremely wide ranging, with certain mutations causing no anemia and others leading to death in utero, childhood, or early adulthood. Thalassemias are named according to the chain with reduced or absent synthesis. Mutations affecting the α - or β -globin gene are most clinically significant because Hb A ($\alpha_2\beta_2$) is the major adult hemoglobin. The decreased or absent synthesis of one of the

chains not only leads to a decreased production of hemoglobin but results in an imbalance in the α/β chain ratio.³ The unaffected gene continues to produce globin chains at normal levels, and the accumulation of the unpaired normal chains damages the red blood cells (RBCs) or their precursors resulting in their premature destruction. This exacerbates the anemia and makes some forms of thalassemia particularly severe.

EPIDEMIOLOGY

The morbidity and mortality due to thalassemia significantly contributes to the global health burden. Approximately 5% of the world's population is a carrier of a clinically significant mutation for a hemoglobinopathy or thalassemia.⁴ Annually an estimated 56,000 infants are born with a form of thalassemia major.^{4,5} Although thalassemia occurs in all parts of the world, its distribution is concentrated in the “thalassemia belt” that extends from the Mediterranean east through the Middle East and India to Southeast Asia and south to Northern Africa (Figure 27-3).⁶ The carrier frequency of β -thalassemia depends on the region, with Sardinia, Cyprus, and Greece having the highest frequency in Europe (6% to 19%) and India, Thailand, and Indonesia having the highest frequency in Asia and Southeast Asia (0.3% to 15%).⁶ The carrier frequency of α -thalassemia varies considerably. In Europe, Cyprus has the highest carrier frequency at 14%.⁶ The carrier frequency reaches 50% to 60% in Eastern Saudi Arabia and parts of Asia and Africa, and may be as high as 75% to 80% in certain groups in Nepal, India, Thailand, and Papua New Guinea.⁶

The geographic location of the thalassemia belt coincides with areas in which malaria is prevalent (Figure 27-3). Thalassemia minor (heterozygous thalassemia) appears to impart

resistance to malaria. This allowed the selective advantage that established thalassemia in high frequency in areas in which malaria is endemic.^{7,8} Several case-control studies evaluated the incidence of thalassemia in patients with severe malaria compared with a control population and consistently found a lower incidence of thalassemia in the population with malaria than in the population without malaria. One study demonstrated that the risk of death from malaria was 40% lower in patients with $\alpha\alpha/-\alpha$ thalassemia and more than 60% lower in those with $-\alpha/-\alpha$ thalassemia.⁸ The mechanism of this resistance is still not fully elucidated; however, two major theories have been put forward: defective growth of the parasite in the affected cell and increased phagocytosis of the infected cell.^{9,10} Although the exact mechanism is not known, the geographic distribution and the case-control studies corroborate the protective nature of the thalassemias in promoting resistance to malaria.

GENETICS OF GLOBIN SYNTHESIS

The normal hemoglobin molecule is a tetramer of two α -like chains (α or ζ) with two β -like chains (β , γ , δ , or ϵ). Combinations of these chains produce six normal hemoglobins. Three are embryonic hemoglobins: Hb Gower-1 ($\zeta_2\epsilon_2$), Hb Gower-2 ($\alpha_2\epsilon_2$), and Hb Portland ($\zeta_2\gamma_2$). The others are fetal hemoglobin (Hb F, $\alpha_2\gamma_2$) and two adult hemoglobins (Hb A, $\alpha_2\beta_2$, and Hb A₂, $\alpha_2\delta_2$). The α -like globin gene cluster is located on chromosome 16, whereas the β -like globin gene cluster is on chromosome 11. The α -like globin gene cluster contains three functional genes: *HBZ* (ζ -globin), *HBA1* (α_1 -globin), and *HBA2* (α_2 -globin).^{11,12} The β -like globin gene cluster contains five functional genes: *HBE* (ϵ -globin), *HBG2* ($^C\gamma$ -globin), *HBG1* ($^A\gamma$ -globin), *HBD* (δ -globin), and *HBB* (β -globin).^{3,11,12} These genes are positioned in the order that corresponds with their developmental stage of expression.¹¹ During the first 2 months of gestation, the genes for the embryonic ζ and ϵ chains are expressed, generating Hb Gower-1 ($\zeta_2\epsilon_2$). Expression of the genes for the α and γ chains begins in the second month of gestation, generating two additional embryonic hemoglobins: Hb Gower-2 ($\alpha_2\epsilon_2$) and Hb Portland ($\zeta_2\gamma_2$). By 10 weeks gestation, the genes for the embryonic ζ and ϵ chains are switched off and silenced, while the genes for α and γ chains are upregulated (called the ζ to α switch on chromosome 16, and the ϵ to γ switch on chromosome 11).¹¹ The γ chains combine with the α chains to make Hb F ($\alpha_2\gamma_2$), the predominant hemoglobin of fetal life. The gene for the β chain is initially activated during the second month of gestation, but β chain production occurs at low levels throughout most of fetal life.¹³ Shortly before birth, however, the expression of the γ -globin gene is downregulated, while expression of the β -globin gene is upregulated (called the γ to β switch), so by 6 months of age and through adult life, Hb A ($\alpha_2\beta_2$) is the predominant hemoglobin. The gene for the δ chain is activated shortly before birth, but owing to its weak promoter, only produces a relatively small amount of δ chain, resulting in a low level of Hb A₂ ($\alpha_2\delta_2$) (Chapter 10 and Figure 10-6).^{3,11} Table 28-1 contains the reference intervals for the normal hemoglobins in adults.

TABLE 28-1 Reference Intervals for Normal Hemoglobins in Adults

Hb A ($\alpha_2\beta_2$)	95%–100%
Hb A ₂ ($\alpha_2\delta_2$)	0%–3.5%
Hb F ($\alpha_2\gamma_2$)	0%–2%

The γ -globin genes code for two globin chains ($^C\gamma$ and $^A\gamma$) that differ at position 136 by a single amino acid (glycine and alanine, respectively).¹³ Both of these globin chains are found in Hb F, with no functional difference identified between them. Similarly, the α -globin gene loci are duplicated on each chromosome 16 and also code for two globin chains (α_1 and α_2). Either of these genes can contribute to the two α -globin chains in the hemoglobin tetramer, and no functional difference has been identified between the two. Interspersed between the functional genes on these chromosomes are four functionless gene-like loci or pseudogenes that are designated by the prefixed symbol ψ . The purpose of these pseudogenes is unknown.¹³ The organization of these genes on chromosomes 16 and 11 is shown in Figure 28-1.

An individual inherits one cluster of the five functional genes on chromosome 11 from each parent. The genotype for normal β chain synthesis is designated β/β . Because two α -globin genes (α_1 and α_2) are inherited on each chromosome 16, a normal genotype is designated $\alpha\alpha/\alpha\alpha$.

CATEGORIES OF THALASSEMIA

The thalassemias are divided into β -thalassemias, which include all the disorders of reduced globin chain production arising from the β -globin gene cluster on chromosome 11, and α -thalassemias, which involve the genes for the α_1 and α_2 chains on chromosome 16. Various deletional and non-deletional mutations can cause each of these disorders, and individuals with similar clinical manifestations are often heterogeneous at the genetic level.^{3,11,13}

The β -thalassemias affect mainly the β chain production but also may involve the δ , $^C\gamma$, $^A\gamma$, and ϵ chains. In the β -thalassemias, β^0 is the designation for the various mutations in the β -globin gene in which no β chains are produced. In the homozygous state (β^0/β^0), an individual does not produce Hb A ($\alpha_2\beta_2$). β^+ is the designation for the various mutations in the β -globin gene that result in a partial deficiency of β chains (5% to 30% of normal) and a decrease in production of Hb A.³ Some mutations in the β -globin gene lead to minimal reductions in β chain production and are associated with mild or silent clinical states. The designation β^{silent} for silent carrier has been used for those mutations. The designation $\delta\beta^0$ is used for mutations in the δ - or β -globin genes in which no δ or β chains are produced. In the homozygous state ($\delta\beta^0/\delta\beta^0$), no Hb A ($\alpha_2\beta_2$) or Hb A₂ ($\alpha_2\delta_2$) are produced. The designation $\delta\beta^{\text{Lepore}}$ indicates a fusion of the δ - and β -globin genes that produces Hb Lepore.

The most common mutations in α -thalassemia are deletions involving the α_1 - and/or α_2 -globin genes.^{11,12} The designation

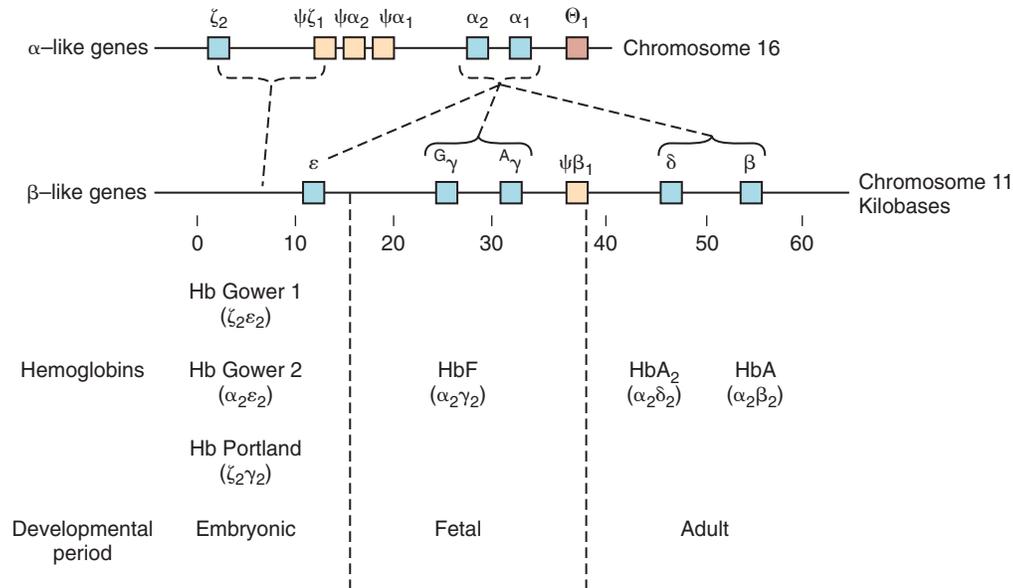


Figure 28-1 Chromosome organization of globin genes and their expression during development. The *light blue boxes* indicate functional globin genes; the *tan boxes* indicate pseudogenes. The scale of the depicted chromosomal segments is in kilobases of DNA. The switch from embryonic to fetal hemoglobin (Hb) occurs by 10 weeks of gestation, and the switch from fetal to adult hemoglobin occurs in the third trimester. (From Cunningham MJ, Sankaran VG, Nathan DG, et al: Chapter 20. The Thalassemias. In: Orkin SH, Nathan DG, Ginsberg D, et al (eds). Nathan and Oski's hematology of infancy and childhood, ed 7, 2009, Saunders, an imprint of Elsevier, page 1017.)

α^+ is used to indicate a deletion of *either* the α_1 - or the α_2 -globin gene on chromosome 16 (also called the $-\alpha$ haplotype). This results in a decreased production of α chains from that chromosome. The designation α^0 is used to indicate a deletion of *both* the α_1 - and α_2 -globin genes on chromosome 16 (also called the $-\alpha$ haplotype). This results in no production of α chains from that chromosome.^{3,11,13} Non-deletional mutations in the α -globin gene can also result in α -thalassemia, but these are less common.¹² The designation α^T is used for these mutations.¹³ The major gene designations in thalassemia are summarized in [Table 28-2](#).

GENETIC DEFECTS CAUSING THALASSEMIA

Types of genetic defects that cause a decrease or absent production of a particular globin chain include single nucleotide (or point) mutations, small insertions or deletions, or large deletions.^{12,13} The mechanisms^{3,11,13} by which these mutations interfere with globin chain production include:

- *Reduced or absent transcription of messenger ribonucleic acid (mRNA)* due to mutations in the promoter region or initiation codon of a globin gene, as well as mutations in polyadenylation sites that decrease mRNA stability
- *mRNA processing errors* due to mutations that add or remove splice sites resulting in no globin chain or altered globin chain production
- *Translation errors* due to mutations that change the codon reading frame (frameshift mutations), substitute an incorrect amino acid codon (missense mutations), add a stop codon causing premature chain termination (nonsense mutations), or remove a stop codon, which results in an elongated

and unstable mRNA that produces a dysfunctional globin chain

- *Deletion of one or more globin genes* resulting in the lack of production of the corresponding globin chains

All of these heterogeneous genetic mutations cause a reduction or lack of synthesis of one or more globin chains, resulting in the thalassemia syndromes ([Figure 28-2](#)).

PATHOPHYSIOLOGY

The clinical manifestations of thalassemia stem from:

1. A reduced or absent production of a particular globin chain, which diminishes hemoglobin synthesis and produces microcytic, hypochromic RBCs; and
2. An unequal production of the α - or β -globin chains causing an imbalance in the α/β chain ratio; this leads to a markedly decreased survival of RBCs and their precursors.^{3,11,13}

The α/β chain imbalance is more significant and determines the clinical severity of the thalassemia.¹¹ The mechanism and the degree of shortened RBC survival are different for the β -thalassemias and α -thalassemias.

Mechanisms in β -Thalassemias

In the β -thalassemias, the unpaired, excess α chains precipitate in the developing RBCs, forming inclusion bodies; this causes oxidative stress and damage to cellular membranes.¹⁴ Apoptosis is triggered, and the damaged and apoptotic RBC precursors are subsequently phagocytized and destroyed in the bone marrow by activated macrophages.¹⁴ In addition, iron accumulation in the RBC precursors (discussed below) and inflammatory cytokines may also contribute to the

TABLE 28-2 Genetic Designations in Thalassemia

Designation	Definition
Designations for Normal β-Globin and α-Globin Genes	
β	Normal β -globin gene; normal amount of β chains produced; one gene located on each chromosome 11
$\alpha\alpha$	Normal α_1 - and α_2 -globin genes on one chromosome (haplotype $\alpha\alpha$); normal amount of α chains produced; two genes located on each chromosome 16
Designations for the Major Thalassemic Genes	
β^0	β -globin gene mutation in which no β chains are produced
β^+	β -globin gene mutation that results in 5% to 30% decrease in β chain production
β^{silent}	β -globin gene mutation that results in mildly decreased β chain production
$\delta\beta^0$	$\delta\beta$ -globin gene deletional or non-deletional mutation in which no δ or β chains are produced; accompanied by some increase in γ chain production
$\delta\beta^{\text{Lepore}}$	$\delta\beta$ -globin gene fusion that produces a small amount of fusion product, hemoglobin Lepore; no δ or β chains are produced; accompanied by some increase in γ chain production
HPFH	Hereditary persistence of fetal hemoglobin; $\delta\beta$ -globin gene deletional or non-deletional mutation in γ -globin gene promoter in which no δ or β chains are produced; accompanied by increase in γ chain production
α^0	Deletion of both α -globin genes on one chromosome (haplotype, $--$) that results in no α chain production
α^+	Deletion of one α -globin gene on one chromosome (haplotype, $-\alpha$) that results in decreased α chain production
α^T	Non-deletional mutation in one α -globin gene on one chromosome (haplotype $\alpha^T\alpha$) that results in decreased α chain production (T denotes thalassemia)

apoptosis.¹⁴ The premature death of RBC precursors in the bone marrow is called *ineffective erythropoiesis*.^{11,14} In this situation, the bone marrow attempts to produce RBCs but is not able to release sufficient viable cells into the circulation. The cells that are released into the periphery are laden with inclusion bodies and are rapidly sequestered and destroyed by macrophages in the spleen (extravascular hemolysis).¹¹ Therefore, in β -thalassemia the anemia is multifactorial and results from ineffective production and increased destruction. Typically, individuals with severe β -thalassemia are asymptomatic during fetal life and through approximately 6 months of age because Hb F ($\alpha_2\gamma_2$) is the predominant circulating hemoglobin. Symptoms usually begin to appear between 6 and 24 months of age, after completion of the γ to β switch.^{3,13,15} To compensate for the decreased expression of the β -globin

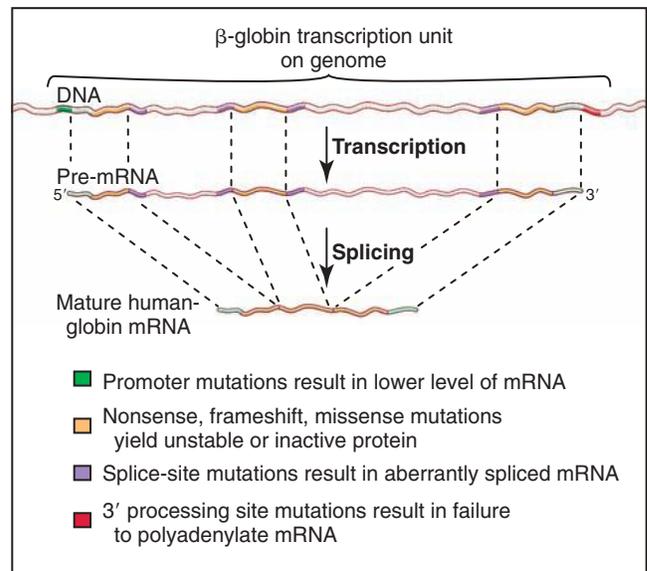


Figure 28-2 The transcription unit of the β -globin gene. The nucleotide sequence of the DNA template is transcribed into a complementary pre-mRNA. The pre-mRNA is processed by removing introns and splicing together the protein coding exons (orange). The DNA sequences required for expression of a functional β -globin chain are indicated in different colors. Mutations in any of these sequences can lead to decreased or absent β -globin chain production. (From Corden JL, Chapter 15. Gene Expression. In Pollard TD, Earnshaw WC. Cell Biology, ed 2. Philadelphia, 2008, Saunders, An imprint of Elsevier, Figure 15-2.)

gene, the γ - and/or δ -globin genes are usually upregulated, but in β -thalassemia major, this increase is insufficient to correct the α/β chain imbalance.³

In β -thalassemia major, the profound anemia stimulates an increase in erythropoietin production by the kidney and results in massive (but ineffective) erythroid hyperplasia.¹¹ In untreated or inadequately treated patients, marked bone changes and deformities occur due to the massive bone marrow expansion. A reduction in bone mineral density and a thinning of the cortex of the bone increases the risk of pathologic fractures.^{3,11} In children, radiographs of the long bones may exhibit a lacy or lucent appearance.³ Skull radiographs may demonstrate a typical “hair on end” appearance due to vertical striations of bony trabeculae (Figure 28-3).^{3,11} A typical facies occurs, with prominence of the forehead (also known as frontal bossing), cheekbones, and upper jaw. Extramedullary erythropoiesis causes hepatosplenomegaly, and foci of hematopoietic tissue can appear in other body areas. Sequestration of blood cells in the enlarged spleen can worsen the anemia and can also cause neutropenia and thrombocytopenia.¹¹ The release of hemoglobin from the excessive destruction of RBCs and their precursors leads to an increase in the level of plasma indirect bilirubin. The bilirubin can diffuse into the tissues, causing jaundice (Chapter 23). Patients also have an increased risk of developing thrombosis.^{3,11}

Iron accumulation in various organs is a serious complication in β -thalassemia major and is a significant cause of

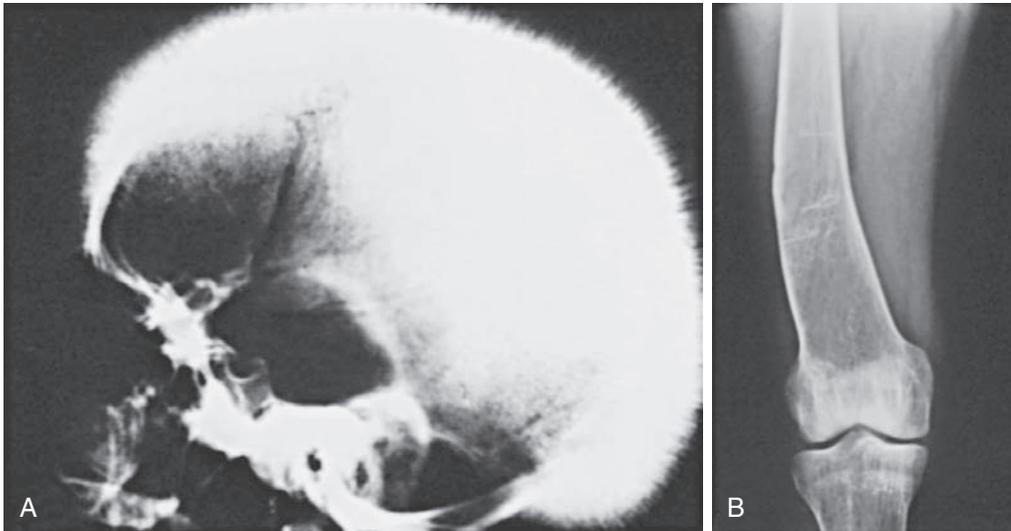


Figure 28-3 Radiologic abnormalities in a patient with homozygous β -thalassemia who receives blood transfusions infrequently (thalassemia intermedia). **A**, Skull radiograph illustrating the typical “hair on end” appearance. **B**, Severe osteoporosis, pseudofractures, thinning of the cortex, and bowing of the femur. (From Cunningham MJ, Sankaran VG, Nathan DG, et al. Chapter 20. The Thalassemias. In: Orkin SH, Nathan DG, Ginsberg D, et al. Nathan and Oski’s hematology of infancy and childhood, ed 7, 2009, Saunders, an imprint of Elsevier.)

morbidity and mortality in adults.³ In children, excess iron causes growth retardation and absence of sexual maturity; in adults, it causes cardiomyopathy, fibrosis and cirrhosis of the liver, and dysfunction of exocrine glands.^{3,15} The risk of organ damage due to iron accumulation begins to increase after 10 to 11 years of age.¹⁵ The iron overload is predominantly due to the regular RBC transfusions required in β -thalassemia major (discussed later). However, the extreme degree of erythropoiesis also suppresses hepcidin production by the liver, resulting in more iron absorption by the intestinal enterocytes (Chapter 11).^{3,16} This increase in intestinal iron absorption further adds to the iron overload burden.^{3,16} The pathophysiology of β -thalassemia major is summarized in Figure 28-4.

Mechanisms in α -Thalassemia

In α -thalassemia, the decreased production of α chains can manifest in utero because the α chain is a component of both fetal and adult hemoglobins. However, the accumulation of non- α chains has different consequences compared to β -thalassemia. In the fetus and newborn, a decrease in production of α chains results in an excess of γ chains. These γ chains accumulate in proportion to the number of deleted or defective α genes.^{11,13} The γ chains are more stable and do not precipitate but instead form hemoglobin tetramers (γ_4) called *Hb Bart*.¹³ After 6 months of age and through adulthood when the γ to β switch is completed, the decrease in α chain production results in excess β chains. The excess β chains are also relatively stable and form tetramers (β_4), called *Hb H*.

Because *Hb H* and *Hb Bart* do not precipitate to any significant degree in the developing RBCs in the bone marrow, patients with α -thalassemia do not have severe ineffective

erythropoiesis.¹¹ As the mature RBCs age in the circulation, however, the β_4 tetramers in *Hb H* eventually precipitate and form inclusion bodies.¹¹ The macrophages in the spleen recognize and remove these abnormal RBCs from the circulation, and the patient manifests a moderate hemolytic anemia.

In addition to the decreased production and shortened RBC survival mechanisms, a third mechanism is involved in the anemia of α -thalassemia. *Hb Bart* and *Hb H* cannot deliver oxygen to tissues due to their very high affinity for oxygen.¹³ A fetus cannot survive with only *Hb Bart* (found with a deletion of all four α -globin genes). The marked tissue hypoxia causes heart failure and massive edema (hydrops fetalis) and hepatomegaly, and the fetus usually dies in utero or shortly after birth.³ This is discussed in the α -thalassemia section later in the chapter.

β -GLOBIN GENE CLUSTER THALASSEMIAS

There is great heterogeneity in the mutations in the β -globin gene cluster that leads to the clinical syndrome of β -thalassemia.¹² More than 300 genetic abnormalities have been discovered, including mutations affecting the β -, δ -, and γ -globin genes individually or in combination.^{12,13} A small subset of mutations, however, accounts for the majority of the mutant alleles within a single ethnic group or geographic area in which β -thalassemia is found.^{3,15} Because multiple mutations are present in each population, most individuals with severe β -thalassemia are compound heterozygotes for two different β -thalassemia mutations.¹¹ A comprehensive list of hemoglobin gene mutations is maintained in the HbVar mutation database, which is available online.¹²

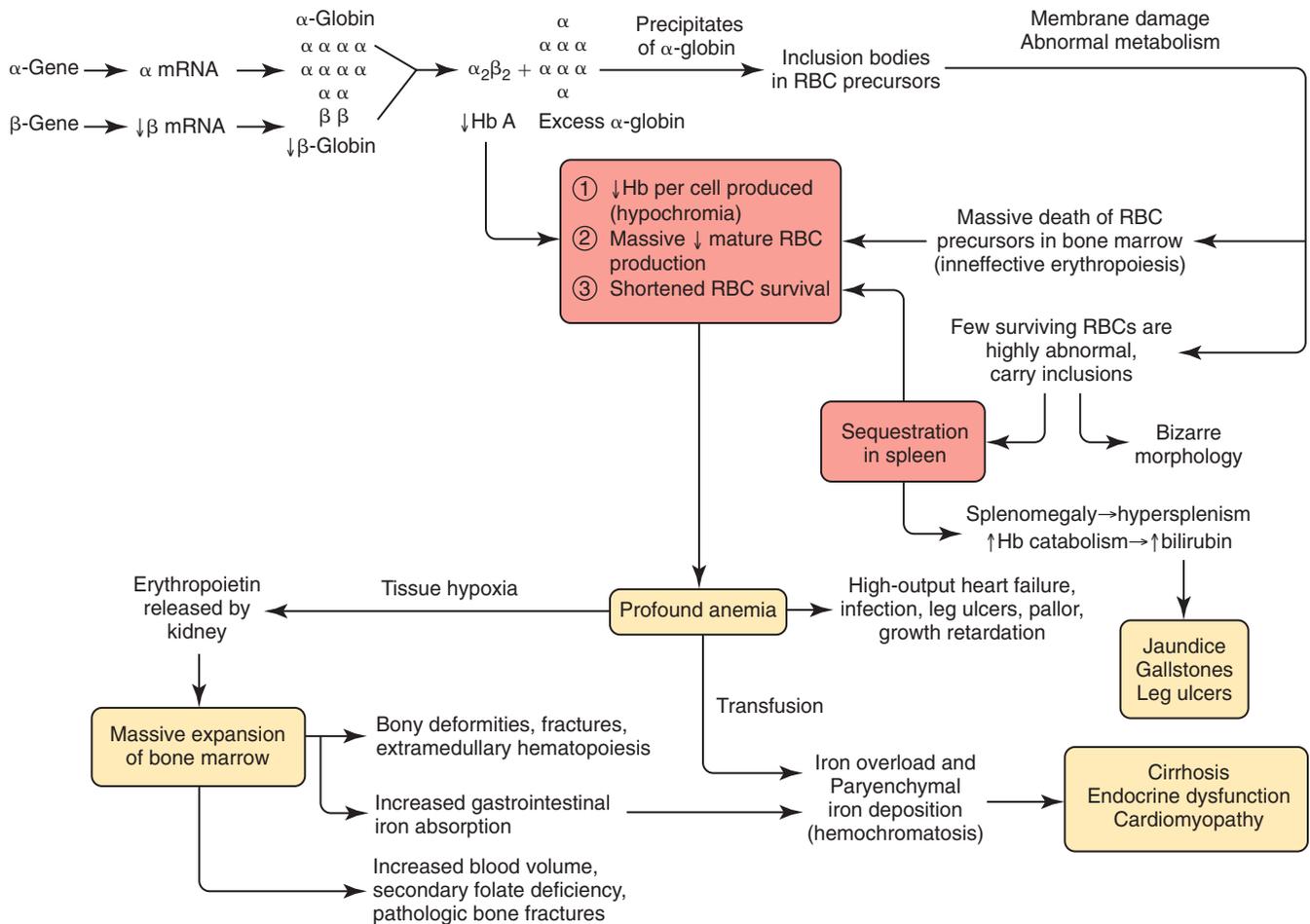


Figure 28-4 Pathophysiology of severe forms of β -thalassemia. The diagram outlines the pathogenesis of clinical abnormalities resulting from the primary defect in β -globin chain synthesis. *RBC*, Red blood cell. (From Giardina PJ, Rivella S: *Thalassemia Syndromes*. In: Hoffman R, Benz EJ, Jr, Silberstein LE, et al, editors: *Hematology: basic principles and practice*, ed 6, Philadelphia, 2013, Saunders, an imprint of Elsevier.)

Clinical Syndromes of β -Thalassemia

β -thalassemia is divided into four categories based on clinical manifestations (Table 28-3).^{3,13}

- β -thalassemia silent carrier (heterozygous state) with no hematologic abnormalities or clinical symptoms
- β -thalassemia minor (heterozygous state) with mild hemolytic anemia, microcytic/hypochromic RBCs, and no clinical symptoms
- β -thalassemia major (homozygous or compound heterozygous state) with severe hemolytic anemia, microcytic/hypochromic RBCs, severe clinical symptoms, and transfusion-dependence
- β -thalassemia intermedia with mild to moderate hemolytic anemia, microcytic/hypochromic RBCs, moderate clinical symptoms, and transfusion independence

The clinical manifestations of the various mutations depend on whether one or both of the β -globin genes are affected and the extent to which the affected gene or genes are expressed. Some mutations result in the complete absence of β chain production, and genes with these mutations are designated as β^0 . Other mutations lead to production of the β chains but at a significantly reduced rate, and these are

designated as β^+ mutations. The range of β chain production in these β^+ mutations varies from 5% to 30% of normal β chain synthesis.³ Still other mutations only minimally reduce β chain production, and genes with those mutations are designated as β^{silent} (Table 28-2).

β -thalassemia is inherited in an autosomal recessive pattern. If both parents are carriers of a β -thalassemia gene mutation, they have a 25% chance of having a child with two mutated β -globin genes (homozygote or compound heterozygote) and clinical manifestations of β -thalassemia major or intermedia.

Silent Carrier State of β -Thalassemia

The designation β^{silent} includes the various heterogeneous β -globin gene mutations that produce only a small decrease in production of the β chains. The silent carrier state ($\beta^{\text{silent}}/\beta$) results in nearly normal α - β chain ratios and no hematologic abnormalities.^{3,11,13} It was first recognized through a study of families in which the affected children had a more severe β -thalassemia syndrome than a parent with typical β -thalassemia minor.¹³ The parents had normal levels of Hb A₂ and a slight microcytosis. Some individuals who are homozygous for a

TABLE 28-3 Clinical Syndromes of β -Thalassemia with Examples of Genotypes

Genotype	Hb A	Hb A ₂	Hb F	Hb Lepore
Normal (Normal Hematologic Parameters)				
β/β	N	N	N	0
Silent Carrier State (Asymptomatic; Normal Hematologic Parameters)				
$\beta^{\text{silent}}/\beta$	N	N	N	0
Thalassemia Minor (Asymptomatic; Mild Hemolytic Anemia; Microcytic, Hypochromic)				
β^+/β	↓	↑	N to Sl ↑	0
β^0/β	↓	↑	N to Sl ↑	0
$\delta\beta^0/\beta$	↓	N to ↓	5%–20%	0
$\delta\beta^{\text{Lepore}}/\beta$	↓	↓	↑	5%–15%
Thalassemia Major (Severe Hemolytic Anemia; Transfusion-Dependent; Microcytic, Hypochromic)				
β^+/β^+	↓↓	V	↑↑	0
β^+/β^0	↓↓↓	V	↑↑	0
β^0/β^0	0	V	↑↑	0
$\delta\beta^{\text{Lepore}}/\delta\beta^{\text{Lepore}}$	0	0	80%	20%
Thalassemia Intermedia (Mild to Moderate Hemolytic Anemia; Transfusion-Independent*; Microcytic, Hypochromic)**				
$\beta^{\text{silent}}/\beta^{\text{silent}}$	↓	↑	↑	0
$\beta^+/\beta^{\text{silent}}$ or $\beta^0/\beta^{\text{silent}}$	↓	↑	↑	0
$\delta\beta^0/\delta\beta^0$	0	0	100%	0
$\beta^0/\delta\beta^0$	0	N	↑↑	0

*Patients who are transfusion-independent do not require regular transfusions for survival, but may need transfusions occasionally, such as during pregnancy or infections.

**Other genotypes are included in this category such as dominantly inherited β -thalassemia (heterozygous for a very severe β -globin gene mutation) and coinheritance of a triplicated α -globin gene ($\alpha\alpha\alpha/\alpha\alpha$) with thalassemia minor.

↑, Increased; ↓, decreased; 0, absent; Hb, hemoglobin; N, normal; Sl, slight; V, variable.

silent thalassemia gene mutation ($\beta^{\text{silent}}/\beta^{\text{silent}}$) have been described.^{13,17} They present with a mild β -thalassemia intermedia phenotype with an increased level of Hb F and Hb A₂.^{13,17}

β -Thalassemia Minor

β -thalassemia minor (also called β -thalassemia trait) results when one β -globin gene is affected by a mutation that decreases or abolishes its expression, whereas the other β -globin gene is normal (heterozygous state). It usually presents as a mild, asymptomatic anemia with hemoglobin ranging from 12.4 to 14.2 g/dL in affected men and 10.8 to 12.8 g/dL in affected women.¹¹ The RBC count is within the reference interval or slightly elevated.^{3,13} The RBCs are microcytic and hypochromic, with a mean cell volume (MCV) less than 75 fL and a

mean cell hemoglobin (MCH) less than 26 pg.³ The reticulocyte count is within the reference interval or slightly increased.³ Some degree of poikilocytosis (including target cells and elliptocytes) and basophilic stippling in the RBCs may be seen on a Wright-stained peripheral blood film (Figure 28-5). The bone marrow shows mild to moderate erythroid hyperplasia, with minimal ineffective erythropoiesis. Hepatomegaly and splenomegaly are seen in a few patients. In the most common β -thalassemia minor syndromes (β^0/β and β^+/β), the Hb A level is 92% to 95% and the Hb A₂ level is characteristically elevated and can vary from 3.5% to 7.0%.^{3,13,15} The Hb F level usually ranges from 1% to 5%.^{3,13} Less common types of β -thalassemia minor exist, such as $\delta\beta^0/\beta$ and $\delta\beta^{\text{Lepore}}/\beta$. Other rare types have atypical features, such as Dutch β^0 -thalassemia minor that shows the expected elevation in Hb A₂ level but an Hb F level in the 5% to 20% range,¹⁸ and another mutant found in a Sardinian family in which the Hb A₂ level is normal.¹⁹

β -Thalassemia Major

β -Thalassemia major is characterized by a severe anemia that requires regular transfusion therapy. It is usually diagnosed between 6 months and 2 years of age (after completion of the γ to β switch) when the child's Hb A level does not increase as expected.^{3,13}

In untreated β -thalassemia major, the hemoglobin level can fall as low as 3 to 4 g/dL.^{3,13} The MCV ranges from 50 to 70 fL.^{11,15} The peripheral blood film shows marked microcytosis, hypochromia, anisocytosis, and poikilocytosis, including target cells, teardrop cells, and elliptocytes. Polychromasia and nucleated red blood cells may be observed (Figure 28-6). RBC inclusions are commonly found, including basophilic stippling, Howell-Jolly bodies, and Pappenheimer bodies, the latter as a result of the excess nonheme iron in the RBCs. The reticulocyte count is only mildly to moderately elevated and is inappropriately lower in relation to the amount of RBC hyperplasia and hemolysis present.³ The inappropriate reticulocytosis results from the apoptosis of RBC precursors in the bone marrow (ineffective erythropoiesis).

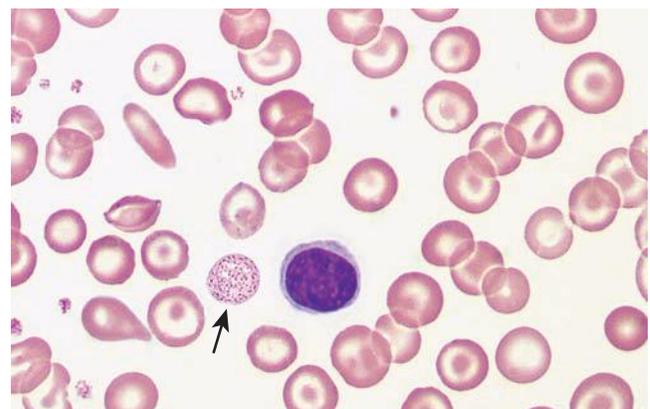


Figure 28-5 Red blood cells (RBCs) from a patient with β -thalassemia minor, showing microcytic, hypochromic RBCs with target cells, other poikilocytes, and basophilic stippling (arrow).

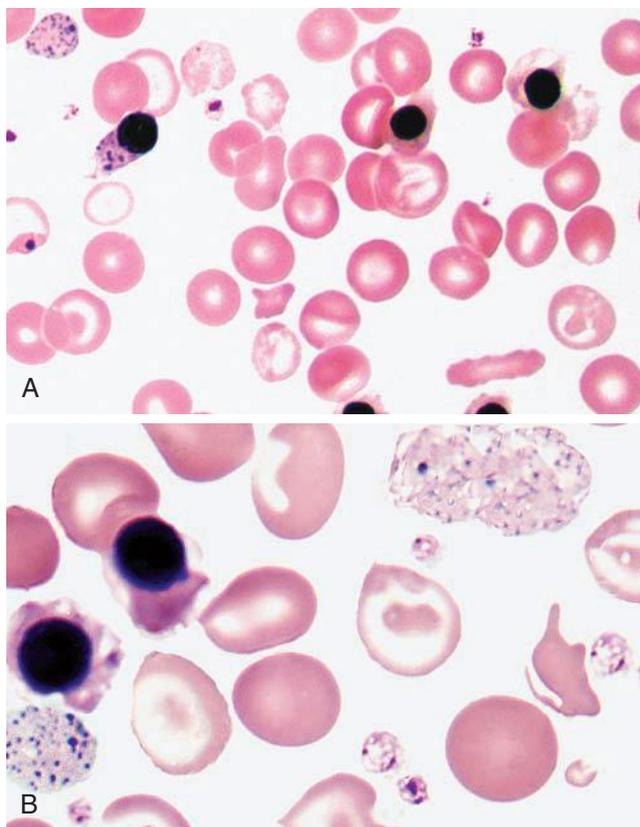


Figure 28-6 Red blood cells from a patient with β -thalassemia major. Note basophilic stippling, microcytosis, hypochromia, target cells, nucleated red blood cells, and red cell fragments. **A**, $\times 500$. **B**, $\times 1000$. (Adapted from Carr JH, Rodak BF: Clinical hematology atlas, ed 4, Philadelphia, 2013, Saunders.)

Hb A is absent or decreased, depending on the specific genotype, which determines whether none (β^0/β^0) or a decreased amount (β^+/β^+ or β^0/β^+) of β chains are produced. Hb A is produced only if a β^+ mutation is present, and usually ranges from 10 to 30%.^{3,15} Hb F ranges from 70% to greater than 90%, depending on the genotype and amount of Hb A.^{13,15} The level of Hb A₂ is variable and can be within or above the reference interval.¹¹ The bone marrow shows marked erythroid hyperplasia, with a myeloid-to-erythroid (M:E) ratio of 1:20 (reference interval is 1.5:1 to 3.3:1). As a result of the massive destruction of erythroid cells and release of free hemoglobin, the serum haptoglobin level is reduced or absent, and the serum lactate dehydrogenase activity is markedly elevated (Chapter 23).

Transfusion therapy is the major therapeutic option for patients with thalassemia major and typically is initiated when the hemoglobin drops to less than 7 g/dL and the patient has clinical symptoms.^{3,20} Typically, 10 to 15 mL/kg of RBCs are transfused every 2 to 5 weeks.³ RBCs that are less than 7 to 10 days old are used for transfusion to allow for maximum donor RBC survival in the patient.³ Typing the patient for the major blood group antigens and transfusing antigen-negative donor RBCs are recommended to reduce the risk of alloimmunization.^{11,15}

Administration of RBC transfusions at regular intervals began in the mid-1970s. The pretransfusion hemoglobin level is usually maintained between 9 and 10.5 g/dL.^{3,21} Such transfusion regimens are termed *hypertransfusion* and are used not only to correct the anemia but to also suppress the marked erythropoiesis. With erythropoiesis suppressed, the marked marrow expansion does not occur, and therefore the bone changes do not take place. In addition, the reduction in erythropoiesis decreases the amount of iron absorbed in the intestinal enterocytes.¹⁶ Children receiving this therapy do not develop hepatosplenomegaly and have much-improved growth and development.³ The transfusion regimens, however, lead to an excess iron burden. Because there is no effective physiologic pathway for iron excretion in the body, the iron contained in the transfused RBCs accumulates in the body. This iron is stored in organs outside the bone marrow (e.g., liver, heart, pancreas), which results in organ damage. The accumulation of iron in the liver leads to cirrhosis, and the deposition of iron in the heart leads to cardiac dysfunction and arrhythmias. In the past, with transfusion therapy alone, thalassemic patients died in their teens, typically from cardiac failure. Now patients undergo iron chelation therapy with the transfusion therapy. Iron chelating agents bind excess iron so that it can be excreted in the urine and stool. The standard chelation therapy is a daily infusion of deferoxamine, usually administered subcutaneously with an infusion pump over 8 to 12 hours.^{3,11} Owing to the cost, inconvenience, and side effects, patients may have poor compliance with the regimen.^{3,11} Two oral iron chelators, deferasirox and deferiprone, have been approved by the U.S. Food and Drug Administration, which may improve compliance, but their long-term efficacy compared to deferoxamine is still being evaluated.^{3,13,22} Additional oral iron chelating drugs are in development. Iron chelation treatment has been able to prevent iron accumulation and the subsequent complications of iron overload, helping to extend life expectancy of patients with β -thalassemia major into the fourth and fifth decade and beyond.^{3,15,22}

Hematopoietic stem cell transplantation (HSCT) is the only curative therapy for thalassemia major.^{3,23} In patients with a good risk profile (on a regular chelation therapy regimen, with no hepatomegaly or liver fibrosis), the average overall and thalassemia-free survival rates are greater than 90% and 80%, respectively.^{15,23} The highest survival rates occur in young patients with an HLA identical sibling donor.²³ Because there is only a 25% chance that a sibling will have the identical HLA genotype, this option is not available to all patients. A well-matched unrelated donor can be used, but survival is not as high, and finding an immunologic match in an unrelated donor is less likely.

Hemoglobin F induction agents, such as hydroxyurea, 5-azacytidine, short chain fatty acids, erythropoietic-stimulating agents, and thalidomide derivatives have been evaluated for therapy in thalassemia major because of their ability to “switch on” the γ -globin gene to produce more γ chains.²⁴ The γ chains then combine with the excess α chains to form Hb F, thus partially correcting the α/β chain imbalance. Hydroxyurea therapy has benefited a few β -thalassemia major patients,

allowing them to become transfusion-independent, but has not been beneficial in the majority of patients.^{3,24} Studies with the other Hb F-inducing agents have shown initial promising results.²⁴ However, larger and better-designed studies are needed to determine the efficacy of these agents in thalassemia major.²⁴

In 2010, a successful lentiviral β -globin gene transfer was reported in an adult with severe Hb E- β^0 -thalassemia who became transfusion-independent.²⁵ Based on this success, clinical trials have opened at multiple sites using lentiviral vectors for β - and γ -globin gene transfer.²⁶ In addition, research on the use of gene therapy to increase expression of the patient's own γ -globin genes has also intensified.²⁶ Ideally, in the future, gene therapy will be able to correct the genetic defect.

β -Thalassemia Intermedia

Thalassemia intermedia is a term used to describe anemia that is more severe than β -thalassemia minor but does not require regular transfusions to maintain hemoglobin level and quality of life (transfusion-independent).^{3,11} Although patients with thalassemia intermedia typically maintain a hemoglobin level greater than 7 g/dL, it is the clinical features rather than the hemoglobin level that determine the diagnosis.^{3,11} In these patients, the α/β chain imbalance falls between that observed in β -thalassemia minor and β -thalassemia major but without the need for regular transfusion therapy. The genotypes of thalassemia intermedia show great heterogeneity. Patients can be homozygous for mutations that cause a mild decrease in β -globin expression. Conversely, they may be compound heterozygous, with one gene causing a mild decrease in β chain production and the other causing a marked reduction in β chain production.^{3,11} In rare instances, only one of the β -globin genes carries a mutation, but it is severe enough to cause a significant anemia. These cases are sometimes called *dominantly inherited* β -thalassemia.¹³ Many of the thalassemia intermedia phenotypes are generated from the coinheritance of one or two abnormal β -globin genes with another hemoglobin defect, such as abnormal α -globin genes or unstable hemoglobins.^{3,11} The coinheritance of α -thalassemia may permit homozygotes with more severe β -thalassemia mutations to remain transfusion independent because the α/β chain ratio is more balanced and fewer free α chains are available to precipitate and cause hemolysis.³ Less severe clinical manifestations also occur when a β -thalassemia mutation is combined with a mutation that increases the expression of the γ -globin gene.³ The increase in Hb F production ($\alpha_2\gamma_2$) helps to compensate for the reduction in Hb A, while helping to correct the α/β balance. Examples of these situations are the deletional forms of $\delta\beta^0$ -thalassemia. Individuals homozygous for these mutations, or compound heterozygotes for $\delta\beta^0$ -thalassemia and a β -thalassemia mutation, have thalassemia intermedia with increased γ chain and Hb F synthesis.^{3,13} Conversely, coinheritance of a triplicated α -globin gene locus ($\alpha\alpha\alpha$) (see section on α -thalassemia) is also a cause of thalassemia intermedia in some individuals heterozygous for β -thalassemia due to the production of more α chains and greater imbalance of the α/β chain ratio.^{3,27}

Because of the genetic heterogeneity of β -thalassemia intermedia, the laboratory and clinical features vary. The degree of anemia and jaundice varies, depending on the extent to the α/β chain imbalance. Because of the presence of splenomegaly, the platelet and neutrophil counts may be low. The clinical course varies from minimal symptoms (despite moderately severe anemia) to severe exercise intolerance and pathologic fractures.¹¹ Patients with thalassemia intermedia also have iron overload even though they do not receive transfusions.³ The markedly accelerated ineffective erythropoiesis suppresses hepcidin production by the liver, which results in more iron absorption by the intestinal enterocytes.¹⁶ Cardiac, liver, and endocrine complications, however, present 10 to 20 years later in thalassemia intermedia patients than in patients who receive regular transfusions.³

Other Thalassemias Caused by Defects in the β -Globin Gene Cluster

Other thalassemias may be caused by deletion, inactivation, or fusion of a combination of genes of the β -globin gene cluster, such as hereditary persistence of fetal hemoglobin (HPFH), $\delta\beta^0$ -thalassemia, and Hb Lepore thalassemia.^{3,11,28}

Thalassemias with Increased Levels of Fetal Hemoglobin

HPFH and $\delta\beta^0$ -thalassemia are closely related, heterogeneous conditions in which Hb F is expressed at increase levels beyond infancy into adulthood. These conditions have similarities but can be differentiated by the clinical presentation, hemoglobin level, MCV, and amount of Hb F produced.¹³

In HPFH, the β -globin gene cluster typically contains a deletion in the $\delta\beta$ region that leads to the increased production of Hb F. However, there are also HPFH conditions that have intact β -globin gene clusters with non-deletional mutations in the promoter region of the γ -globin genes that lead to the increased Hb F production.^{11,13,29} Because individuals with these mutations are characteristically asymptomatic, this condition is of little significance except when it interacts with other forms of thalassemia or structural hemoglobin variants, such as Hb S. The additional γ chains produced are able to replace the missing β chains and help to restore the balance of α and non- α chains (γ or β). Significant variation is seen in heterozygotes for deletional-type HPFH, but these patients typically are asymptomatic with a normal MCV and Hb F levels of 10% to 35%, depending on the mutation.^{3,11} Homozygotes for deletional-type HPFH are also asymptomatic. They have a normal to slightly increased hemoglobin level, 100% Hb F, with slightly hypochromic and microcytic RBCs.¹³ The increase in hemoglobin observed in some patients is likely a response to the slight hypoxia induced by the higher oxygen affinity of Hb F compared with Hb A.³ When assessed using the Kleihauer-Betke acid elution stain (discussed later), the distribution of Hb F in HPFH is usually pancellular (deletional-types), but it can be heterocellular (non-deletional-types). In contrast, the Hb F distribution in the other β -globin gene cluster thalassemias is always heterocellular.^{3,30}

The $\delta\beta^0$ -thalassemias are also characterized by deletions in the δ - and β -globin genes and an increase in Hb F in adult life. Non-deletional types have also been described.¹¹ In this condition, however, the increase in production of the γ chains is not sufficient to completely restore the balance between the α and non- α chains. Heterozygous $\delta\beta^0$ -thalassemia individuals ($\delta\beta^0/\beta$) have a decreased level of Hb A, normal or decreased level of Hb A₂, and 5% to 20% Hb F.^{11,13} They have a β -thalassemia minor phenotype, with a slight decrease in hemoglobin level and hypochromic, microcytic RBCs. Homozygous $\delta\beta^0$ -thalassemia individuals ($\delta\beta^0/\delta\beta^0$) have hypochromic, microcytic RBCs, 100% Hb F, and a β -thalassemia intermedia phenotype.^{11,13} (Table 28-3).

Hemoglobin Lepore Thalassemia

Hemoglobin Lepore ($\delta\beta^{\text{Lepore}}$) is structural variant and rare type of $\delta\beta$ -thalassemia caused by a fusion of the $\delta\beta$ -globin genes.¹² This mutation occurs during meiosis due to nonhomologous crossover between the δ -globin locus on one chromosome and the β -globin locus on the other chromosome. The Lepore globin chain expressed by the $\delta\beta$ fusion gene contains the first 22 to 87 amino acids of the N-terminus of the δ chain and the last 31 to 97 amino acids of the C-terminus of the β chain, depending on the variant.¹² The $\delta\beta$ fusion gene produces a reduced level of the Lepore globin chain because its transcription is under the control of the δ -globin gene promoter, which is much less active than the β -globin gene promoter.³ Conversely, in the reciprocal fusion on the other chromosome (called *anti-Lepore*), the β -globin gene locus is intact, so normal production of the β chain occurs.^{3,13} In heterozygotes ($\delta\beta^{\text{Lepore}}/\beta$), there is a decreased level of Hb A and Hb A₂, an increase in Hb F, and approximately 5% to 15% Hb Lepore.^{3,13} The clinical manifestations are similar to β -thalassemia minor. In homozygotes ($\delta\beta^{\text{Lepore}}/\delta\beta^{\text{Lepore}}$), there are no normal δ - or β -globin genes, no production of Hb A and Hb A₂, and approximately 80% Hb F and 20% Hb Lepore.¹³ The clinical manifestations are similar to β -thalassemia major³ (Table 28-3).

Screening for β -Thalassemia Minor

Because of the high carrier frequency of β -thalassemia mutations worldwide, screening has become an important global health issue.^{5,6} Mass-screening programs in Italy and Greece combined with prenatal diagnosis have led to a significant reduction in the number of children born with β -thalassemia major.¹³ Carrier parents have a 25% risk of having a child with thalassemia major or thalassemia intermedia, depending on the particular β globin gene mutations.¹² Potential carriers of these disorders can be initially identified by measuring the hemoglobin level, MCV, and the Hb A₂ and Hb F levels.^{13,31} Other causes of microcytic anemias, such as iron deficiency, need to be ruled out. Molecular genetic testing of the *HBB* gene is performed for carrier detection in couples seeking preconception counseling and prenatal testing.¹⁵

α -THALASSEMIAS

In contrast to β -thalassemia, in which point mutations in the β -globin gene cluster are the most common type of mutation, in

α -thalassemia large deletions involving the α_1 - and/or α_2 -globin genes are the predominant genetic defect.¹¹⁻¹³ Non-deletional mutations (mostly point mutations) also occur in α -thalassemia but are uncommon.^{12,13,32} The extent of decreased production of the α chain depends on the specific mutation, the number of α -globin genes affected, and whether the affected α -globin gene is α_2 or α_1 .¹¹ The α_2 -globin gene produces approximately 75% of the α chains in normal RBCs, so mutations in the α_2 -globin gene generally cause more severe anemia than mutations affecting the α_1 -globin gene.^{11,13,32} The notation for the normal α -globin gene complex or haplotype is $\alpha\alpha$, which signifies the two normal genes (α_2 and α_1) on one chromosome 16. A normal genotype is $\alpha\alpha/\alpha\alpha$.

The α -thalassemias are divided into two haplotypes: α^0 -thalassemia and α^+ -thalassemia. In the α^0 -thalassemia haplotype (originally named α -thal-1), a deletion of both α -globin genes on chromosome 16 results in no α chain production from that chromosome. The designation, $-$, is used for the α^0 -thalassemia haplotype.^{11,13} There are 21 known mutations that produce the α^0 -thalassemia haplotype and involve deletion of both of the α -globin genes or the entire α -globin gene cluster (including the ζ -globin gene) on one chromosome.¹² The α^0 haplotype ($-$) is found in approximately 4% of the population in Southeast Asia, is found less frequently in the Mediterranean region, and occurs infrequently in other parts of the world.⁶

In the α^+ -thalassemia haplotype (originally named α -thal-2), a deletional or non-deletional mutation in one of the two α -globin genes on chromosome 16 results in decreased α chain production from that chromosome.¹¹ The designation, $-\alpha$, is used for the deletional mutations, while the designation, $\alpha^T\alpha$, is used for the non-deletional mutations. The deletional α^+ haplotype ($-\alpha$) is by far the most common of the α -thalassemia haplotypes. It is widely distributed throughout the thalassemia belt and central Africa (Figure 27-3), with a carrier frequency reaching 50% to 80% in some regions of Saudi Arabia, India, Southeast Asia, and Africa.⁶ The deletional α^+ haplotype ($-\alpha$) is also found in about 30% of African Americans.³² The non-deletional α^+ haplotype ($\alpha^T\alpha$) is relatively uncommon.^{13,32} More than 40 different mutations are known, the majority of which are point mutations that affect the predominant α_2 gene.¹² The $\alpha^T\alpha$ haplotype produces unstable α chains or fewer α chains than in the $-\alpha$ haplotype and generally results in a more severe anemia.^{13,32}

One of the most common non-deletional α -globin gene mutations is Constant Spring ($\alpha_2^{142\text{Stop}\rightarrow\text{Gln}}$), also called α^{CS} haplotype, $\alpha^{\text{CS}}\alpha$.^{12,13} It is the result of a point mutation in the α_2 -globin gene that changes the stop codon at 142 to a glutamine codon.^{12,13,32} As a result, additional bases are added to the end of the mRNA during transcription until the next stop codon is reached. The elongated mRNA is very unstable and produces only a small amount of the α^{CS} chain.^{3,33,34} The α^{CS} chains (with an additional 31 amino acids added to the C-terminal end) combine with β chains to form Hb Constant Spring, but the incorporation of a longer α chain makes the tetramer unstable.³⁴ Because of the instability of both the mRNA and the Hb tetramer, the circulating level of Hb Constant Spring is very low.^{13,32,34} Consequently, hemoglobin

Constant Spring is difficult to detect by alkaline hemoglobin electrophoresis, and when present, is visualized as a faint, slow-moving band near the point of origin.¹³

Clinical Syndromes of α -Thalassemia

Four clinical syndromes are present in α -thalassemia, depending on the gene number, *cis* or *trans* pairing, and the amount of α chains produced.^{11,13} The four syndromes are^{11,13} (Table 28-4):

- Silent carrier state
- α -thalassemia minor
- Hb H disease
- Hb Bart hydrops fetalis syndrome

Silent Carrier State

The deletion of one α -globin gene, leaving three functional α -globin genes ($-\alpha/\alpha\alpha$), is the major cause of the silent carrier

TABLE 28-4 Clinical Syndromes of α -Thalassemia

Genotype	Hb A	Hb Bart (in Newborn)	Hb H (in Adult)	Hb Constant Spring
Normal (Normal Hematologic Parameters)				
$\alpha\alpha/\alpha\alpha$	N	0	0	0
Silent Carrier State (Asymptomatic; Normal Hematologic Parameters)				
$-\alpha/\alpha\alpha$	N	1%–2%	0	0
$\alpha^{CS}\alpha/\alpha\alpha$	N	1%–3%	0	<1%
α-Thalassemia Minor (Asymptomatic; Mild Hemolytic Anemia; Microcytic, Hypochromic)				
$-\alpha/\alpha\alpha$	SI ↓	5%–15%	0	0
$-\alpha/-\alpha$	SI ↓	5%–15%	0	0
$\alpha^{CS}\alpha/\alpha^{CS}\alpha^*$	SI ↓	5%–15%	0	<6%
Hb H Disease (Mild to Moderate Hemolytic Anemia; Transfusion-Independent**; Microcytic, Hypochromic)				
$-\alpha/-\alpha$	↓	10%–40%	1%–40%	0
$-\alpha/\alpha^{CS}\alpha^{\dagger}$	↓	↑↑	↑↑	<1%
Hb Bart Hydrops Fetalis Syndrome (Severe Anemia; Usually Infants are Stillborn or Die Shortly after Birth)				
$---$	0	80%–90% (remainder Hb Portland)	NA	0

* $\alpha^{CS}\alpha/\alpha^{CS}\alpha$ genotype results in mild to moderate hemolytic anemia with jaundice and hepatosplenomegaly.

**Patients who are transfusion-independent do not require regular transfusions for survival, but may need transfusions occasionally, such as during pregnancy or infections.

\dagger $-\alpha/\alpha^{CS}\alpha$ genotype and other non-deletional genotypes ($-\alpha/\alpha^T\alpha$) result in Hb H disease that is moderate to severe and may require more frequent transfusions than the deletional $-\alpha/-\alpha$ genotype.

↓, Decreased; ↑↑ increased more than $-\alpha/-\alpha$; 0, absent; <, less than; CS, Constant Spring; Hb, hemoglobin; N, normal; NA, not applicable.

state. The α/β chain ratio is nearly normal, and no hematologic abnormalities are present.^{11,13} Because one α -globin gene is absent, there is a slight decrease in α chain production. There is a slight excess of γ chains at birth that form tetramers of Hb Bart (γ_4) in the range of 1% to 2%.^{11,13} There is no reliable way to diagnose silent carrier state other than genetic analysis. A non-deletional α^+ mutation in one α -globin gene ($\alpha^T\alpha/\alpha\alpha$) also results in the silent carrier state. In the heterozygous mutation, $\alpha^{CS}\alpha/\alpha\alpha$, Hb Constant Spring is less than 1% of the total hemoglobin.¹³

α -Thalassemia Minor

Deletion of two α -globin genes is the major cause of α -thalassemia minor. It exists in two forms: homozygous α^+ ($-\alpha/-\alpha$) or heterozygous α^0 ($-\alpha/\alpha$).^{11,13} This syndrome is asymptomatic and characterized by a mild anemia (typical hemoglobin concentration is 12 to 13 g/dL) with microcytic, hypochromic RBCs. At birth, the proportion of Hb Bart is in the range of 5% to 15%.¹³ In adults, the production of α and β chains is balanced, so Hb H (β_4) is not usually present. Homozygosity for non-deletional mutations in both α_2 -globin genes ($\alpha^T\alpha/\alpha^T\alpha$) produces a mild to moderate hemolytic anemia, often with jaundice and hepatosplenomegaly.^{13,34} In the homozygous mutation, $\alpha^{CS}\alpha/\alpha^{CS}\alpha$, Hb Constant Spring is 5% to 6% of the total hemoglobin and the hemoglobin concentration is 9 to 11 gm/dL.^{11,13,34}

Hemoglobin H Disease

Deletion of three α -globin genes is the major cause of Hb H disease in which only one α -globin gene remains to produce α chains ($-\alpha/-\alpha$).^{11,13} This genetic abnormality is particularly common in Asians because of the prevalence of the α^0 gene haplotype ($---$). It is characterized by the accumulation of excess unpaired β chains that form tetramers of Hb H in adults. In the newborn, Hb Bart comprises 10% to 40% of the hemoglobin, with the remainder being Hb F and Hb A. After the γ to β switch, Hb H replaces most of the Hb Bart, so Hb H is in the range of 1% to 40%, with a reduced amount of Hb A₂, traces of Hb Bart, and the remainder Hb A.^{11,13,32,33} The non-deletional α^+ haplotype, when combined with the α^0 haplotype ($-\alpha/\alpha^T\alpha$), generally produces a more severe Hb H disease with a higher level of Hb H than the α^0 interaction with the deletional α^+ haplotype ($-\alpha/-\alpha$).^{13,32,34} Hb H-Hb Constant Spring ($-\alpha/\alpha^{CS}\alpha$) is an example.^{13,34-36}

Hb H disease is characterized by a mild to moderate, chronic hemolytic anemia with hemoglobin concentrations averaging 7 to 10 g/dL, and reticulocyte counts of 5% to 10%, although a wide variability in clinical and laboratory findings exists.^{11,36} The bone marrow exhibits erythroid hyperplasia, and the spleen is usually enlarged. Patients with deletional Hb H disease are transfusion-independent, that is, they do not require regular transfusions. However, infection, pregnancy, or exposure to oxidative drugs may cause a hemolytic crisis requiring transfusions on a temporary basis.

Hemolytic crises often lead to the detection of the disease because individuals with Hb H disease may otherwise be asymptomatic. The RBCs are microcytic and hypochromic, with marked poikilocytosis, including target cells and bizarre

shapes. Hb H is vulnerable to oxidation and gradually precipitates in the circulating RBCs to form inclusion bodies of denatured hemoglobin.¹¹ These inclusions alter the shape and viscoelastic properties of the RBCs, contributing to the decreased RBC survival. Splenectomy is beneficial in patients with markedly enlarged spleens.³² When incubated with brilliant cresyl blue or new methylene blue, RBCs with Hb H display fine, evenly distributed, granular inclusions. These inclusions are typically removed as the RBC passes through the spleen. Before splenectomy, only a portion of the cells have this characteristic, but after the spleen is removed, most of the RBCs are full of these inclusions. These cells are often described as “golf balls” or “raspberries” (Figure 28-7).

Two distinct conditions are associated with Hb H disease and congenital physical and intellectual abnormalities: alpha-thalassemia retardation-16 (ATR-16) syndrome and alpha-thalassemia X-linked intellectual disability (ATRX) syndrome. Patients with the ATR-16 syndrome inherit or acquire a large deletion in the short arm of chromosome 16, which removes the ζ - and α -globin genes as well as all the flanking genes to the terminus of the chromosome.³³ Patients have physical deformities, intellectual disabilities, and Hb H disease.^{32,33,35} The ATRX syndrome is due to mutations of the ATRX gene located on the X chromosome.^{37,38} The ATRX protein is a component of a large complex that regulates expression of various genes, including the α -globin genes.^{33,38} The regulation is accomplished by DNA remodeling and/or methylation, thus affecting the transcription, replication, and repair of the target genes.^{32,33,36,38} Therefore, when the ATRX gene is mutated, patients have decreased α chain production.^{32,33,35,38} Affected males with ATRX syndrome have pronounced intellectual disability, physical deformities, developmental delay, and Hb H disease. An acquired Hb H disease with mutations in the ATRX gene has been found in myelodysplastic syndrome.^{33,38,39}

Hb Bart Hydrops Fetalis Syndrome

Homozygous α^0 -thalassemia ($-/-$) results in the absence of all α chain production and usually results in death in utero or

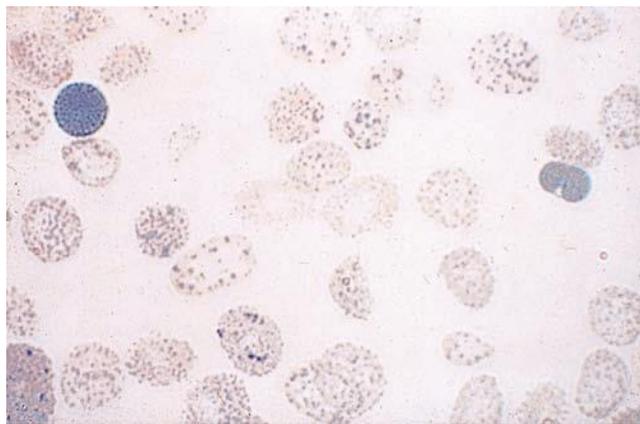


Figure 28-7 Red blood cells from a patient with hemoglobin H disease, incubated with brilliant cresyl blue, which have acquired fine, evenly dispersed granular inclusions and “golf ball” appearance. (From the American Society for Hematology slide bank.)

shortly after birth.^{3,11} The fetus is severely anemic, which leads to cardiac failure and edema in the fetal subcutaneous tissues (hydrops fetalis). Hb Bart (γ_4) is the predominant hemoglobin, along with a small amount of Hb Portland ($\zeta_2\gamma_2$) and traces of Hb H.^{3,11} Hb Bart has a very high oxygen affinity; it does not deliver oxygen to the tissues.^{11,13} The fetus can survive until the third trimester because of Hb Portland, but this hemoglobin cannot support the later stages of fetal growth, and the affected fetus is severely anoxic.¹¹ The fetus is delivered prematurely and is usually stillborn or dies shortly after birth. In addition to anemia, edema, and ascites, the fetus has gross hepatosplenomegaly and cardiomegaly.^{3,11} At delivery, there is a severe microcytic, hypochromic anemia (hemoglobin concentration of 3 to 8 gm/dL) with numerous nucleated RBCs in the peripheral blood.³³ The bone marrow cavity is expanded, and marked erythroid hyperplasia is present, along with foci of extramedullary erythropoiesis.

Hydropic pregnancies are hazardous to the mother, resulting in toxemia and severe postpartum hemorrhage.¹¹ Hydropic changes are detected in midgestation by means of ultrasound testing.⁴⁰ If both parents carry one α^0 -thalassemia haplotype ($-/-\alpha\alpha$), prenatal diagnosis of homozygosity can be made by molecular genetic testing of fetal cells from chorionic villus sampling or amniotic fluid.³³ Absence of the α -globin genes establishes the diagnosis. Early termination of the pregnancy prevents the serious maternal complications.¹¹

THALASSEMIA ASSOCIATED WITH STRUCTURAL HEMOGLOBIN VARIANTS

Hemoglobin S-Thalassemia

Sickle cell anemia (Hb SS)- α -thalassemia is a genetic abnormality due to the coinheritance of two abnormal β -globin genes for Hb S and an α -thalassemia haplotype. Hb SS- α^+ -thalassemia is fairly common because the genes for Hb S and the α^+ -thalassemia haplotype, $-\alpha$, are common in populations of African ancestry. Individuals with Hb SS- α^+ -thalassemia have a milder anemia with higher hemoglobin levels and lower reticulocyte counts than those with sickle cell anemia alone.⁴¹ In one study, Hb SS individuals with the genotypes, $\alpha\alpha/\alpha\alpha$, $-\alpha/\alpha\alpha$, and $-\alpha/-\alpha$, had average hemoglobin concentrations of 8.4, 9.0, and 9.5 g/dL, respectively, and reticulocyte counts of 10.8%, 8.8%, and 6.9%, respectively.⁴¹

Hb S- β -thalassemia is a compound heterozygous condition that results from the inheritance of a β -thalassemia gene from one parent and an Hb S gene from the other. This syndrome has been reported in the populations of Africa, the Mediterranean area, the Middle East, and India.¹³ The clinical expression of Hb S- β -thalassemia depends on the type of β -thalassemia mutation inherited.^{11,13} Individuals with Hb S- β^+ -thalassemia produce variable amounts of normal β chains. Patients have mostly Hb S with slightly elevated Hb A₂ and variable amounts of Hb F and Hb A, depending on the specific abnormal β^+ gene inherited. These patients can be distinguished from those with sickle cell anemia by the presence of microcytosis, splenomegaly, an elevated Hb A₂ level, and an Hb A level that is less than the Hb S level.

The interaction of β^{silent} -thalassemia (in which β chains are produced at mildly reduced levels) and Hb S results in a condition that may be slightly more severe than sickle cell trait. Typically, there is mild hemolytic anemia with splenomegaly. These patients can be distinguished from patients with sickle cell trait by the presence of microcytosis and splenomegaly. Hemoglobin electrophoresis or HPLC confirms this condition when the quantity of Hb S exceeds that of Hb A. In sickle cell trait, Hb A is the predominant hemoglobin.

The combination of β^0 -thalassemia and Hb S produces a phenotype similar to sickle cell anemia with a similar incidence of stroke and a similar life expectancy.⁴² Both conditions lack Hb A and produce severe painful crises as the predominant symptom. Typically, the microcytosis and elevated Hb A₂ level in Hb S- β^0 -thalassemia distinguish it from sickle cell anemia.

Hemoglobin C-Thalassemia

Hb C- β -thalassemia produces moderately severe hemolysis, splenomegaly, hypochromia, microcytosis, and numerous target cells. The hemoglobin electrophoresis pattern varies, depending on the type of β -thalassemia gene defect, with higher Hb C concentrations in patients when there is minimal or no β chain production.¹³

Hemoglobin E-Thalassemia

Hb E- β -thalassemia is a significant concern in Southeast Asia and Eastern India owing to the high prevalence of both genetic mutations.¹³ Hb E is due to a point mutation that inserts a splice site in the β -globin gene, and results in decreased production of Hb E.³ In the homozygous state (Hb EE) the clinical symptoms are similar to a mild β -thalassemia. (Chapter 27) When the mutations are coinherited in the compound heterozygous state, there is a marked reduction of β chain production. The clinical symptoms are similar to β -thalassemia intermedia or β -thalassemia major, depending on the particular β -globin gene mutation.¹³ Table 28-5 summarizes some compound

heterozygous states of β -thalassemia combined with a structural β -globin defect.

DIAGNOSIS OF THALASSEMIA

History and Physical Examination

Individual and family histories are paramount in the diagnosis of thalassemia. The ethnic background of the individual should be investigated because of the increased prevalence of specific gene mutations in certain populations. In the clinical examination, findings that suggest thalassemia include pallor (due to the anemia); jaundice (due to the hemolysis); splenomegaly (caused by sequestration of the abnormal RBCs, excessive extravascular hemolysis, and some extramedullary erythropoiesis); and skeletal deformities (due to the massive expansion of the bone marrow cavities). These findings are particularly prominent in untreated or partially treated β -thalassemia major.¹³ Table 28-6 contains a summary of tests for the diagnosis of thalassemia.

Laboratory Methods

Complete Blood Count with Peripheral Blood Film Review

Although most thalassemias result in a microcytic and hypochromic anemia, laboratory results can vary from borderline abnormal to markedly abnormal; this depends on the type and number of globin gene mutations. The hemoglobin and hematocrit are decreased, but the RBC count can be disproportionately high relative to the degree of anemia, which can generate a very low MCV and mean cell hemoglobin (MCH). The mean cell hemoglobin concentration (MCHC) is also decreased. The RBC distribution width (RDW) is elevated (reflecting anisocytosis) in untreated β -thalassemia major, but it is often normal in β -thalassemia minor. On a Wright-stained peripheral blood film, the RBCs are typically microcytic and hypochromic, except in the silent carrier

TABLE 28-5 β -Thalassemia Associated with Structural β -Globin Variants (Compound Heterozygotes)

Genotype	Hb A	Hb A ₂	Hb F	Other Hb	RBC Morphology	Clinical Manifestations	Treatment
Hb S- β^+ -thalassemia	↓↓	↑	N to ↑	Hb S > Hb A	Microcytes, sickle cells, target cells	Ranges from mild to severe anemia with recurrent vasoocclusive crises	Ranges from no treatment to transfusion support and pain control
Hb S- β^0 -thalassemia	0	↑	N to ↑	Hb S			
Hb C- β^+ -thalassemia	↓↓	†	↑	Hb C > Hb A	Microcytes, Hb C crystals, target cells	Ranges from moderate to severe anemia	Usually no treatment needed
Hb C- β^0 -thalassemia	0	†	↑	Hb C			
Hb E- β^+ -thalassemia	↓↓	†	↑↑	Hb E > Hb A	Microcytes, target cells	Ranges from mild to severe anemia with transfusion dependency	Ranges from no treatment to transfusion support
Hb E- β^0 -thalassemia	0	†	↑↑	Hb E			

↑, Increased; ↓, decreased; 0, absent; Hb, hemoglobin; N, normal; RBC, red blood cell.

*Clinical manifestations depend on the amount of Hb A produced; compound heterozygotes with the β^0 gene have more severe symptoms.

†Not all methods can quantitate Hb A₂ in the presence of the abnormal hemoglobin. High-performance liquid chromatography can separate Hb A₂ from Hb C; capillary zone electrophoresis can separate Hb A₂ from Hb E.

TABLE 28-6 Laboratory Diagnosis of Thalassemias^{15,31,33,43}

Screening tests	Complete blood count	HGB, HCT, MCV, MCH, MCHC: ↓
	Peripheral blood film review	RETIC: sl to mod ↑ Varying degrees of microcytosis, hypochromia, target cells, anisocytosis, poikilocytosis, RBC inclusions, NRBCs
	Iron studies (to rule out IDA)	Serum ferritin and serum iron: N or ↑; TIBC: N
Presumptive diagnosis	Supravital stain	α-thal: Hb H inclusions
	Hemoglobin fraction quantification by electrophoresis, HPLC, and/or CZE	β-thal: Hb A ↓ or 0; Hb A ₂ ↑ (carriers); Hb F: usually ↑; Hb Lepore; other mutants α-thal: Hb A: ↓ or 0 (hydrops fetalis); Hb A ₂ ↓; Hb Bart, Hb H, Hb Constant Spring, other mutants
Definitive* diagnosis	Molecular genetic tests:	Step 1: Targeted mutation analysis:
	β-thal: >250 mutations** in <i>HBB</i>	β-thal: initial screen for four to six most common mutations if specific ethnic group known
	α-thal: >100 mutations** in <i>HBA1</i> and/or <i>HBA2</i>	α-thal: initial screen for seven most common deletions
		If negative, Step 2: DNA sequence analysis If negative, Step 3: Deletion/duplication analysis (e.g., MLPA, aCGH)

*Required for prenatal diagnosis, preconception risk assessment/carrier detection in couples, diagnosis of rare or complex mutations, determining prognosis in young children,

**From reference 12 (HbVar database, accessed May 10, 2014).

aCGH, Array comparative genomic hybridization; CZE, capillary zone electrophoresis; Hb, hemoglobin; HCT, hematocrit; HGB, hemoglobin level (g/dL); IDA, iron deficiency anemia; MCH, mean cell hemoglobin; MCHC, mean cell hemoglobin concentration; MCV, mean cell volume; MLPA, multiplex ligation-dependent probe amplification; mod, moderate; RETIC, reticulocyte count; RBCs, red blood cells; NRBCs, nucleated red blood cells; sl, slight; thal, thalassemia.

phenotypes, in which the RBCs appear normal. In β-thalassemia minor, α-thalassemia minor, and Hb H disease, the cells are microcytic with target cells and slight to moderate poikilocytosis. In homozygous and compound heterozygous β-thalassemia, extreme poikilocytosis may be present, including target cells and elliptocytes, in addition to polychromasia, basophilic stippling, Howell-Jolly bodies, Pappenheimer bodies, and nucleated RBCs.

Reticulocyte Count

The reticulocyte count is elevated, which indicates that the bone marrow is responding to a hemolytic process. In Hb H disease, the typical reticulocyte count is 5% to 10%.¹³ In homozygous β-thalassemia, it is typically 2% to 8%, disproportionately low relative to the degree of anemia.¹³ An inadequate reticulocytosis reflects the ineffective erythropoiesis.

Supravital Staining

In α-thalassemia minor, Hb H disease, and silent carrier α-thalassemia, brilliant cresyl blue or new methylene blue stain may be used to induce precipitation of the intrinsically unstable Hb H.^{35,43} Hb H inclusions (denatured β-globin chains) typically appear as small, multiple, irregularly shaped greenish-blue bodies that are uniformly distributed throughout the RBC. They produce a pitted pattern on the RBCs similar to the pattern of a golf ball or raspberry (Figure 28-7). In Hb H disease, almost all RBCs contain Hb H inclusions.³⁵ In α-thalassemia minor, only a few cells may contain these inclusions, and in silent carrier α-thalassemia, only a rare cell does. These inclusions appear different from Heinz bodies, which are larger and fewer in number and most often appear eccentrically along the inner membrane of the RBC. This test is very sensitive in detecting Hb H in the α-thalassemia conditions.⁴³

Assessment of Normal and Variant Hemoglobins

The major clinical laboratory methods used to identify and quantify normal and variant hemoglobins include hemoglobin electrophoresis, cation-exchange high-performance liquid chromatography (HPLC), and capillary zone electrophoresis (CZE).⁴⁴ Each of these methods has advantages and limitations, and no one method is able to identify and quantify all hemoglobins. Therefore, a combination of at least two of the above methods is used for confirmation of a hemoglobin variant.⁴⁵ Molecular genetic testing is required to detect specific mutations in globin genes and definitively identify the type of thalassemia or hemoglobinopathy. Molecular genetic testing is not usually required in adults with typical findings on the CBC, electrophoresis, and/or HPLC, but it is required for prenatal diagnosis, preconception risk assessment/carrier detection in couples, diagnosis of rare or complex mutations, and determining prognosis in young children.^{15,31,33,44}

Hemoglobin electrophoresis at alkaline pH has been the traditional tool for thalassemia and hemoglobinopathy diagnosis. In this method, patient RBC lysate is spotted on a solid support (such as agarose) and subjected to an electrical current in an alkaline buffer. Normal and variant hemoglobins will migrate and separate on the support according to their charge. The support is stained, and each hemoglobin band is quantified by scanning densitometry and reported as a percentage of the total hemoglobin.⁴⁵ This technique is able to distinguish the common hemoglobins, such as Hb A, Hb F, Hb S, Hb C, and the fast-moving hemoglobins, Hb H and Hb Bart.⁴⁴⁻⁴⁶ Electrophoresis, however, has several limitations: it is labor-intensive and cannot accurately quantify Hb A₂ and Hb F. In addition, Hb S and Hb C must be confirmed by another method because Hb D and Hb G comigrate with Hb S, and Hb E and Hb O^{Arab} comigrate with Hb C.⁴⁵ Methods used for confirmation usually include agar electrophoresis at acid pH,

HPLC, or CZE, or in the case of Hb S, the solubility test (Figures 27-6, 27-7, 27-8, 27-9). Figure 28-8 shows the relative hemoglobin mobilities in alkaline electrophoresis for various thalassemias and hemoglobinopathies.

In HPLC, patient RBC lysate in buffer is injected into a cation-exchange column. Both normal and variant hemoglobins will bind to the column. An elution buffer is injected and forms a gradient of varying ionic strength.⁴⁵ The various hemoglobin types will be differentially eluted from the column, each having a specific column retention time. As each hemoglobin fraction passes near the end of the column, a detector measures the absorbance of the fraction at 415 nm, which is recorded as a peak on a chromatogram.⁴⁵ The area under the peak is used to quantify the hemoglobin fraction, which is reported as a percentage of total hemoglobin. With the availability of fully automated instruments, HPLC has replaced hemoglobin electrophoresis in many laboratories as the routine screening method for analysis of hemoglobins.⁴⁴ The method is ideal for thalassemia screening because it can accurately and quickly quantitate Hb A, Hb A₂, and Hb F with 100% sensitivity and 90% specificity if no hemoglobin variants are present (Figure 28-9).⁴⁶ The precise and accurate quantification of Hb A₂ is particularly important in screening individuals for β -thalassemia minor (trait). HPLC can also presumptively identify and quantify hemoglobin variants even in low concentration.^{44,45} HPLC, however, requires specialized instrumentation and extensive experience and training to accurately interpret the complex chromatograms.^{44,45,47} Additional limitations of HPLC include the following: Hb A₂ and Hb E have the same retention time and therefore cannot be accurately quantified by this method; Hb A₂ can be overestimated in the presence of Hb S due to overlapping peaks and underestimated in the presence of Hb D^{Punjab}, and it is not able to identify all variants.⁴⁴⁻⁴⁷ A manual microcolumn method is also available for the measurement of Hb A₂.⁴⁵

In capillary zone electrophoresis (CZE), patient RBC lysate is introduced into a thin silica glass capillary tube in an alkaline buffer. When a current is applied, the various hemoglobin fractions migrate to the cathode at different velocities due to electro-osmotic flow.⁴⁵ As each hemoglobin fraction passes near the end of the capillary, a detector measures the absorbance of the fraction at 415 nm, which is recorded as a peak on an electrophoretogram. The instrument calculates the

percentage of each hemoglobin fraction using an integration of the area under the peak and the migration time.⁴⁵ Fully automated systems are available that provide rapid and accurate identification and quantification. The peaks are placed into zones in the electrophoretogram for easier identification, and it can presumptively identify hemoglobin variants, including those in low concentration (Figure 28-9).⁴⁵ An advantage of CZE over HPLC is that it can separate and quantify Hb A₂ in the presence of Hb E.⁴⁴ However, because there is overlap in the peaks for Hb A₂ and Hb C, it cannot quantify Hb A₂ in the presence of Hb C.⁴⁴ As with HPLC, it also cannot detect all variants.^{44,45} Complementing electrophoresis, HPLC, and/or CZE results, however, have minimized the limitations of all these methods.⁴⁷ Other technologies such as isoelectric focusing and mass spectrometry are used in newborn screening programs for detection of common hemoglobin variants.⁴⁴⁻⁴⁵

Molecular Genetic Testing

For mutations in the *HBB* gene, targeted mutation analysis using polymerase chain reaction (PCR)-based methods can be initially performed for detection and quantification of the four to six most common mutations if an individual's ethnicity is known.^{3,15} This strategy allows a mutation detection rate of 91% to 95% in Mediterranean, Middle East, Thai, and Chinese populations, and 75% to 80% in African and African-American populations.¹⁵ In multiethnic individuals or if the ethnicity is unknown, DNA sequencing of the *HBB* gene is performed including exons, intervening sequences, splice sites, and 5' and 3' untranslated regulatory regions.^{15,46} This strategy enables detection of approximately 95% of known mutants.^{15,31} If sequencing is not successful, testing can reflex to deletion/duplication analysis (such as multiplex ligation-dependent probe amplification or array-based comparative genomic hybridization) (Chapter 31).^{15,31,46}

For mutations in the *HBA1* or *HBA2* genes, PCR-based targeted mutation analysis can also be initially performed for the seven most common deletional mutations.³³ This strategy detects approximately 90% of all alleles, but the detection rate varies by method.^{33,36} If the above screening is not successful, DNA sequencing of the *HBA1* and *HBA2* genes or deletion/duplication analysis can be performed as described above.³³

	N	SCT	SCA	S- β^+T	S- β^0T	β^+T	β^0T	βTT	$\delta\beta^0T$	HPFH
A	■	■	—	—	—	—	—	■	—	■
F	—	—	—	—	—	—	—	—	■	—
S	—	■	■	■	■	—	—	—	—	—
C, A ₂	—	—	—	—	—	—	—	—	—	—

Figure 28-8 Relative electrophoretic mobilities on cellulose acetate (pH 8.4) of various hemoglobins (Hbs) important in the diagnosis of thalassemia syndromes and hemoglobinopathies. β^0T , β^0 -thalassemia major, β^0/β^0 (no Hb A, increased Hb F, slight increase in Hb A₂); β^+T , β^+ -thalassemia major, β^+/β^+ (decreased Hb A, increased Hb F, slight increase in Hb A₂); βTT , β -thalassemia minor (slight decrease in Hb A, increased Hb A₂, some Hb F); $\delta\beta^0T$, $\delta\beta^0$ -thalassemia, homozygous, $\delta\beta^0/\delta\beta^0$ (100% Hb F); *HPFH*, hereditary persistence of fetal hemoglobin, heterozygous (mostly Hb A, some Hb F, no Hb A₂); *N*, normal; *SCA*, sickle cell anemia (no Hb A, mostly Hb S, increased Hb F, normal Hb A₂); *SCT*, sickle cell trait (Hb A > Hb S, normal Hb A₂ and F); *S- β^0T* , sickle cell- β^0 -thalassemia (no Hb A, increased Hb A₂ and F, mostly Hb S); *S- β^+T* , sickle cell- β^+ -thalassemia (Hb A < Hb S, increased Hb A₂ and F).

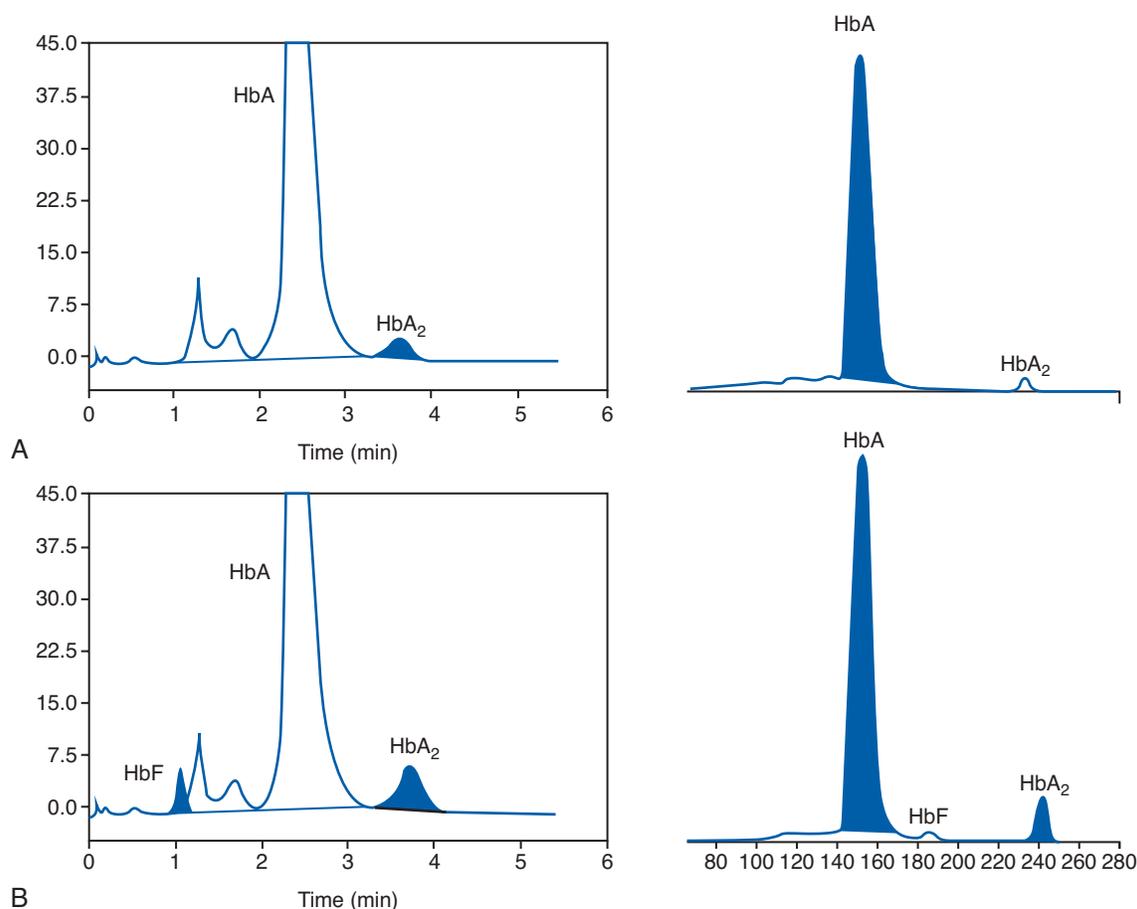


Figure 28-9 Separation and quantification of hemoglobin fractions by high performance liquid chromatography (Bio-Rad, left) and capillary electrophoresis (Sebia, right). **A**, Healthy adult with Hb F < 1% and Hb A₂ < 3.5%; **B**, Adult with β -thalassemia minor with increased Hb F and Hb A₂. (Modified from Giordano PC: Strategies for basic laboratory diagnostics of the hemoglobinopathies in multiethnic societies: interpretation of results and pitfalls. *Int Jnl Lab Hem* 35: 465-479, 2013, Figure 3, p. 472.)

When the parents' mutation is known, analysis for the specific mutation in fetal cells can be done on specimens from amniocentesis (at 15 to 18 weeks' gestation), chorionic villus sampling (at 10 to 12 weeks' gestation), or with preimplantation genetic diagnosis using a cell from a 3-day-old embryo after in vitro fertilization.^{15,33}

Other Procedures

The classic *alkali denaturation* test is accurate and precise to quantify Hb F in the 0.2% to 50% range.⁴⁸ Most human hemoglobins are denatured on exposure to a strong alkali, but Hb F is not. The Hb F can be separated and its concentration compared with that of other hemoglobins. Consistent methodology is required to ensure accurate results.⁴⁸ However, automated HPLC is now often used to quantify Hb F.^{45,46}

In the Kleihauer-Betke acid elution slide test, peripheral blood films are ethanol-fixed and immersed in a citrate-acid buffer (pH 3.3). Adult hemoglobins are eluted from the RBCs, whereas Hb F resists acid elution and remains in the cell. When the cells are subsequently stained, RBCs containing Hb F will take up the stain, whereas RBCs containing only adult hemoglobin will appear as "ghosts." This test determines if the Hb F

distribution in RBCs is pancellular (found in all RBCs in deletional HPFH cases) or heterocellular (found in some but not all RBCs in β -globin gene cluster thalassemias and non-deletional HPFH cases).^{35,45} The Kleihauer-Betke slide test is also used to estimate the volume of fetal-maternal hemorrhage to determine if an increased dose of Rh immune globulin is needed for an Rh-negative mother who delivers an Rh-positive baby. Because the Kleihauer-Betke slide test is cumbersome to perform and results are difficult to replicate, flow cytometry is becoming the standard test to measure fetal-maternal hemorrhage quickly and accurately.⁴⁹

In underdeveloped countries with limited technology, a single-tube osmotic fragility test has been used to screen populations for thalassemia carriers.¹¹ This is based on the fact that carriers have hypochromic RBCs, resulting in decreased osmotic fragility.^{46,50} An aliquot of anticoagulated blood is incubated in 0.375% saline for 5 minutes.⁴⁴ Because the solution is hypotonic, normal RBCs will lyse and the solution will clear. However, patients with thalassemia have hypochromic RBCs that will not lyse in 0.375% saline, and the solution will remain turbid. This test is not specific for thalassemia and will be positive for any condition causing hypochromia, including iron deficiency anemia.

Differential Diagnosis of Thalassemia Minor and Iron Deficiency Anemia

The RBCs in thalassemia minor are microcytic and hypochromic, and this disease must be differentiated from iron deficiency anemia and other microcytic, hypochromic anemias. The differential diagnosis for microcytic, hypochromic anemias is relatively limited (Table 20-1). Differentiating thalassemia minor from iron deficiency is important to avoid unnecessary tests or treatments. An incorrect presumption that a patient has iron deficiency may lead to inappropriate iron therapy or to unnecessary diagnostic procedures, such as a colonoscopy, to identify a source of blood loss.

Clinical history is crucial. A family history of thalassemia raises the suspicion for this diagnosis. A history of previously normal hemoglobin levels and RBC indices, significant bleeding, or pica leads to the diagnosis of iron deficiency.⁵¹ Pica means cravings for nonfood items such as clay, dirt, or starch. The most common pica symptom in the United States is pagophagia, the craving to chew on ice.⁵¹

Iron deficiency and β -thalassemia minor are best differentiated using serum ferritin level, serum iron level, total iron-binding capacity, transferrin saturation, and Hb A₂ level, along with a complete blood count (CBC) and examination of a peripheral blood film.^{51,52} Additional testing may also include soluble transferrin receptor and zinc protoporphyrin levels (Chapter 20).⁵¹

Before evaluating Hb A₂ levels for β -thalassemia minor, iron deficiency should be ruled out. Low iron levels in patients with β -thalassemia minor decrease the Hb A₂ levels.⁵² The iron stores need to be replenished before the laboratory analysis for thalassemia is undertaken.

A mild erythrocytosis (high RBC count) and marked microcytosis (low MCV) are found more commonly in β -thalassemia minor. In iron deficiency anemia, the RBC count and MCV may be normal or decreased, depending on whether the deficiency is developing or long-standing.⁵³⁻⁵⁵ The RDW can be normal or increased in both β -thalassemia minor and iron deficiency anemia, with a significant overlap of values; therefore, the RDW alone cannot distinguish these conditions.⁵²⁻⁵⁴ Various discrimination indices have been proposed to distinguish β -thalassemia minor from iron deficiency anemia using a calculation based on the RBC count, hemoglobin level, MCV, MCH, and/or RDW (such as the Mentzer, Green and King, England and Fraser, Shine and Lal, and Srivastava indices).⁵²⁻⁵⁴ Unfortunately, the sensitivity of these indices in discriminating β -thalassemia minor and iron deficiency anemia ranged from 60% to 96% in various studies, which leads to a high number of false-negative results. Thus their use for screening is not appropriate.^{3,11,51-53} The peripheral blood film may demonstrate basophilic stippling in β -thalassemia minor, which can distinguish it from iron deficiency. Because target cells can be found in both conditions, however, their presence does not help discriminate between the two disorders.

SUMMARY

- Thalassemias are a group of heterogeneous disorders in which one or more globin chains are reduced or absent.
- Thalassemias result in a hypochromic, microcytic anemia due to decreased production of hemoglobin. The imbalance of globin chain synthesis causes an excess of the normally produced globin chain that damages the RBCs or their precursors and results in hemolysis.
- β -Thalassemia is caused by mutations that affect the β -globin gene complex. It is clinically manifested as silent carrier state, thalassemia minor, thalassemia intermedia, or thalassemia major.
- In the silent carrier state ($\beta^{\text{silent}}/\beta$), the blood picture is completely normal. β -thalassemia minor (β^0/β or β^+/β) is a mild, asymptomatic, microcytic, hypochromic anemia; it is usually characterized by an elevated Hb A₂ level, which aids in diagnosis. β -thalassemia major is a severe anemia leading to transfusion dependence. β -thalassemia intermedia manifests abnormalities with a severity between those of β -thalassemia major and β -thalassemia minor, and does not require regular transfusions.
- The α -thalassemias are usually caused by a deletion of one, two, three, or all four of the α -globin genes, resulting in reduced or absent production of α chains.
- In α -thalassemias, tetramers of γ chains form Hb Bart in the fetus and newborn, and tetramers of β chains form Hb H in the adult.
- The α -thalassemias are divided clinically into silent carrier state, α -thalassemia minor, Hb H disease, and Hb Bart hydrops fetalis syndrome.
- Silent carrier α -thalassemia is a result of the deletion, or rarely a non-deletional mutation, of one of four α -globin genes ($-\alpha/\alpha\alpha$) or ($\alpha^T\alpha/\alpha\alpha$); it is associated with a normal RBC profile and is asymptomatic. α -Thalassemia minor is a result of the deletion of two α -globin genes ($-\alpha/-\alpha$ or $-/\alpha\alpha$) and is clinically similar to β -thalassemia minor except that Hb A₂ is not increased.
- Hb H disease is a result of the deletion of three of the four α -globin genes ($-/-\alpha$); Hb H inclusions (β_4) precipitate in older circulating RBCs, causing a hemolytic anemia. The RBCs are microcytic and hypochromic, and the disease is clinically similar to β -thalassemia intermedia. In Hb Bart hydrops fetalis syndrome, all four of the α -globin genes are deleted ($-/-/-$). There is severe anemia, and fetal death usually occurs in utero or shortly after birth. The predominant hemoglobin is Hb Bart (γ_4).
- The preliminary diagnosis of thalassemia is made from the complete blood count results and RBC morphology, hemoglobin electrophoresis, high-performance liquid chromatography, or capillary zone electrophoresis. Molecular genetic testing is required for definitive diagnosis.
- Thalassemia trait must be differentiated from other microcytic, hypochromic anemias, especially iron deficiency anemia. Iron studies are important for this differentiation.

Now that you have completed this chapter, go back and read again the case study at the beginning and respond to the questions presented.

REVIEW QUESTIONS

Answers can be found in the Appendix.

1. The thalassemias are caused by:
 - a. Structurally abnormal hemoglobins
 - b. Absent or defective synthesis of a polypeptide chain in hemoglobin
 - c. Excessive absorption of iron
 - d. Abnormal or defective protoporphyrin synthesis
2. Thalassemia is more prevalent in individuals from areas along the tropics because it confers:
 - a. Heat resistance to those heterozygous for a thalassemia gene
 - b. Selective advantage against tuberculosis
 - c. Selective advantage against malaria
 - d. Resistance to mosquito bites
3. The hemolytic anemia associated with the thalassemias is due to:
 - a. Imbalance of globin chain synthesis
 - b. Microcytic, hypochromic cells
 - c. Ineffective erythropoiesis caused by immune factors
 - d. Structurally abnormal hemoglobin
4. β -Thalassemia minor (heterozygous) usually exhibits:
 - a. Increased Hb Constant Spring
 - b. 50% Hb F
 - c. No Hb A
 - d. Increased Hb A₂
5. RBC morphologic features in β -thalassemia would most likely include:
 - a. Microcytes, hypochromia, target cells, elliptocytes, stippled cells
 - b. Macrocytes, acanthocytes, target cells, stippled cells
 - c. Microcytes, sickle cells
 - d. Macrocytes, hypochromia, target cells, stippled cells
6. The predominant hemoglobin present in β^0 -thalassemia major is:
 - a. Hb A
 - b. Hb A₂
 - c. Hb F
 - d. Hb C
7. Heterozygous HPFH is characterized by:
 - a. 10% to 35% Hb F with normal RBC morphology
 - b. 100% Hb F with slightly hypochromic, microcytic cells
 - c. A decreased amount of Hb F with normal RBC morphology
 - d. 5% to 15% Hb F with hypochromic, macrocytic cells
8. Hb H is composed of:
 - a. Two α and two β chains
 - b. Two ϵ and two γ chains
 - c. Four β chains
 - d. Four γ chains
9. Hb Bart is composed of:
 - a. Two α and two β chains
 - b. Two ϵ and two γ chains
 - c. Four β chains
 - d. Four γ chains
10. When one α gene is deleted (α -/ $\alpha\alpha$), a patient has:
 - a. Normal hemoglobin levels
 - b. Mild anemia (hemoglobin range 9 to 11 g/dL)
 - c. Moderate anemia (hemoglobin range 7 to 9 gm/dL)
 - d. Marked anemia requiring regular transfusions
11. In which part of the world is the α gene mutation causing Hb Bart hydrops fetalis (- -/- -) most common?
 - a. Northern Africa
 - b. Mediterranean
 - c. Middle East
 - d. Southeast Asia
12. The condition Hb S- β^0 -thalassemia has a clinical course that resembles:
 - a. Sickle cell trait
 - b. Sickle cell anemia
 - c. β -Thalassemia minor
 - d. β -Thalassemia major
13. Hb H inclusions in a supravital stain preparation appear as:
 - a. A few large, blue, round bodies in the RBCs with aggregated reticulum
 - b. Uniformly stained blue cytoplasm in the RBC
 - c. Small, evenly distributed, greenish-blue granules that pit the surface of RBCs
 - d. Uniform round bodies that adhere to the RBC membrane
14. Which of the following laboratory findings is *inconsistent* with β -thalassemia minor?
 - a. A slightly elevated RBC count and marked microcytosis
 - b. Target cells and basophilic stippling on the peripheral blood film
 - c. Hemoglobin level of 10 to 13 g/dL
 - d. Elevated MCHC and spherocytic RBCs
15. A 4-month-old infant of Asian heritage is seen for a well-baby check. Because of pallor, the physician suspects anemia and orders a CBC. The RBC count is $4.5 \times 10^9/L$, Hb concentration is 10 g/dL, and MCV is 77 fL, with microcytosis, hypochromia, poikilocytosis, and mild polychromasia noted on the peripheral blood film. These findings should lead the physician to suspect:
 - a. β -Thalassemia major
 - b. α -Thalassemia silent carrier state
 - c. Iron deficiency anemia
 - d. Homozygous α -thalassemia (- -/- -)

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Nonmalignant Leukocyte Disorders 29

Steven Marionneaux

OUTLINE

Qualitative Disorders of Leukocytes

Morphologic Abnormalities with and without Functional Defects

Normal Morphology with Functional Abnormalities

Monocyte/Macrophage Lysosomal Storage Diseases
Genetic B and T Lymphocyte Abnormalities

Quantitative Abnormalities of Leukocytes

Neutrophils

Eosinophils

Basophils

Monocytes

Lymphocytes

Qualitative (Morphologic) Changes

Neutrophils

Monocytes

Lymphocytes

Infectious Mononucleosis

OBJECTIVES

After the completion of this chapter, the reader will be able to:

1. Describe the basic genetic defect and the morphologic consequences in Pelger-Huët anomaly.
2. Discuss how Pelger-Huët cells might be confused with the presence of a neutrophilic left shift.
3. Compare and contrast two genetic causes of neutrophilic hypersegmentation.
4. Describe the basic genetic defect and the morphologic consequences in Alder-Reilly anomaly, Chédiak-Higashi syndrome, and May-Hegglin anomaly.
5. Indicate how inclusions in Alder-Reilly anomaly and May-Hegglin anomaly might be confused with morphologically similar conditions.
6. Discuss the defect and functional consequences of chronic granulomatous disease.
7. Describe the cellular deficiencies and functional consequences of leukocyte adhesion disorders.
8. Describe the characteristic macrophage morphology associated with the mucopolysaccharidoses, Gaucher disease, and Niemann-Pick disease.
9. Describe the basic defect in genetic disorders leading to decreased T lymphocyte production, decreased B lymphocyte production, and the combined decrease of T, B, and natural killer lymphocytes.
10. Define what is meant by *neutrophilia*, *neutropenia*, *lymphocytosis*, *lymphocytopenia*, *monocytosis*, *monocytopenia*, *eosinophilia*, *eosinopenia*, and *basophilia*, and give some examples of conditions in which each occurs.
11. Describe the nonmalignant alterations in granulocyte, monocyte, and lymphocyte morphology that are associated with infection, inflammation, or other causes.
12. Outline pathogenesis; and clinical/laboratory features of infectious mononucleosis.

CASE STUDIES

After studying the material in this chapter, the reader should be able to respond to the following case studies:

Case 1

A 5-year-old boy has a long history of recurring infections, including gastroenteritis, pneumonia, severe staphylococcal infections, and a liver abscess. He was treated with antibiotics in each case and responded well, albeit slowly. The CBC was essentially normal, and no morphologic abnormalities were detected. His neutrophils were tested and were shown to migrate normally and to respond to chemotactic agents. His neutrophils also phagocytized normally; however, they were not able to reduce nitroblue tetrazolium to its insoluble formazan.

1. What is the most likely cause of this child's recurring infections?
2. Genetically and biochemically speaking, what is the specific nature of the problem?
3. What is the prognosis as it relates to treatment?
4. How is this disorder transmitted genetically in the majority of cases?

Case 2

A 66-year-old retired male professor presents with malaise, weakness, a fever of 102° F, anorexia, and weight loss. Blood cultures $\times 8$ were negative for pathogens. Ova and parasite examinations $\times 5$ produced negative findings. Tuberculosis and fungal serologic testing were negative. The CBC revealed a mild normocytic, normochromic anemia; a WBC count of $4.0 \times 10^9/L$; and a platelet count of $130 \times 10^9/L$. The differential count showed a neutrophilic

Continued

CASE STUDIES—cont'd

After studying the material in this chapter, the reader should be able to respond to the following case studies:

left shift, 20% lymphocytes, 28% monocytes, and 1% basophils. Neutrophils contained toxic granulation and large vacuoles. Monocytes were large and highly vacuolated. The patient's erythrocyte sedimentation rate was 70 mm/hour.

1. Based on the differential results, what cells should the medical laboratory scientist look for on the patient's blood film?

2. Where on the blood film should the examination be made and why?
3. What is the significance if the suspected cells are found?
4. What preparation other than a blood film might be helpful in this situation?

This chapter concentrates on nonmalignant disorders of WBCs—etiologies underlying changes in number and morphology of neutrophils, lymphocytes, monocytes, eosinophils, and basophils. Both hereditary and acquired causes are presented. In recent years, the genetic origin of many of these disorders has come to light and the chapter has been updated to reflect these findings.

QUALITATIVE DISORDERS OF LEUKOCYTES

Morphologic Abnormalities with and without Functional Defects

Pelger-Huët Anomaly

Pelger-Huët anomaly (PHA), also known as true or congenital PHA, is an autosomal dominant disorder characterized by decreased nuclear segmentation (bilobed, unilobed) and a characteristic coarse chromatin clumping pattern potentially affecting all leukocytes, although morphologic changes are most obvious in mature neutrophils.¹ The prevalence of PHA is approximately 1 in 4785 in the United States.¹ The disorder is a result of a mutation in the *lamin* β -receptor gene.² The *lamin* β receptor is an inner nuclear membrane protein that combines β -type lamins and heterochromatin and plays a major role in leukocyte nuclear shape changes that occur during normal maturation.¹ Mutations in the *lamin* β -receptor gene result in the morphologic changes characteristic of PHA, although the exact pathological mechanisms are not known.¹ The nuclei may appear round, ovoid, or peanut shaped. Bilobed forms—the characteristic spectacle-like (“pince-nez”) morphology with the nuclei attached by a thin filament—can also be seen (Figure 29-1).³ In homozygous PHA, all neutrophils are affected and demonstrate round nuclei, whereas in the heterozygote, 55% to 93% of the neutrophil population are affected, and there is generally a mixture of all of the aforementioned nuclear shapes.⁴ Neutrophils in Pelger-Huët anomaly appear to function normally.⁵

Pseudo- or Acquired Pelger-Huët Anomaly

Neutrophils with PHA morphology can be observed in patients with hematologic malignancies such as myelodysplastic syndromes (MDS), acute myeloid leukemia, and chronic myeloproliferative neoplasms. Pseudo-PHA neutrophils can also be seen in patients with HIV infection,

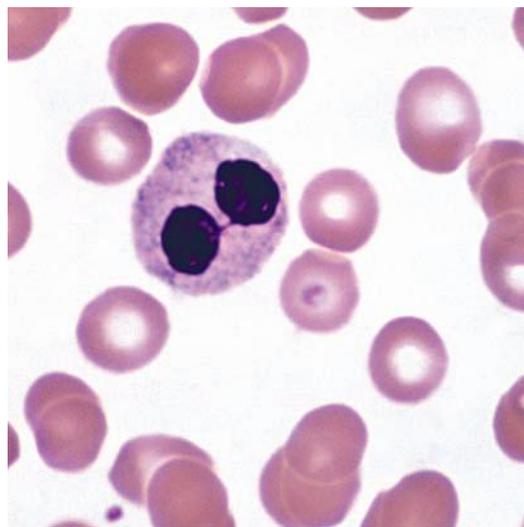


Figure 29-1 Pelger-Huët cell. Pince-nez form with two rounded segments connected by a filament. Notice the dense chromatin pattern.

tuberculosis, *Mycoplasma pneumoniae* and severe bacterial infections. Drugs known to induce pseudo-PHA include mycophenolate mofetil, valproate, sulfisoxazole, ganciclovir, ibuprofen, and chemotherapies such as paclitaxel and docetaxel.⁶

Laboratory Issues in Pelger-Huët Anomaly (True/Congenital and Pseudo/Acquired)

Differentiating between true PHA and pseudo-PHA can be challenging. (1) An important consideration is the number of cells present with PHA morphology. In true PHA, the number of affected cells is much higher than in pseudo-PHA (63% to 93% vs. <38%, respectively).^{4,7} (2) Also in true PHA, all WBC lineages are potentially affected in terms of nuclear shape and chromatin structure. (3) In pseudo-PHA the phenomenon is usually seen only in neutrophils, except for some cases of MDS where monocytes, eosinophils, and basophils may exhibit PHA morphology. (4) Furthermore, if true PHA is suspected, a careful examination of peripheral blood smears of family members may reveal similar findings. (5) Hypogranular neutrophils are a common finding in MDS-related pseudo-PHA. In true PHA, neutrophils exhibit normal granulation.

In both true and pseudo-PHA there are potential challenges for the clinical lab related to cell identification. Because the

nuclei of Pelger-Huët neutrophils may appear round, oval, or peanut shaped, the cells may be classified and counted as myelocytes, metamyelocytes, or band neutrophils, mimicking a neutrophilic left shift and triggering a clinical workup to uncover the cause. A careful examination of the chromatin structure can help to differentiate between Pelger-Huët cells, which are mature, and neutrophils, which are less mature.² Also, immature neutrophils such as metamyelocytes and myelocytes should show some degree of cytoplasmic basophilia. PHA cells are mature, so the cytoplasm is nearly colorless, except for the color imparted by normal cytoplasmic granulation.

Another laboratory challenge is determining the most appropriate label to use for reporting Pelger-Huët cells. PHA neutrophils may be unilobed or bilobed, so “segmented neutrophil” seems inappropriate. “Band neutrophil” is also not suitable for reasons stated above. It is suggested that one label should be applied to all morphologic variants of PHA neutrophils. Laboratories should address this concern and develop standardized labels to be used for all morphologic variants of PHA, the goal being to ensure that the clinician understands that PHA cells are present and that lineage maturity is not left shifted. One suggested approach would be to count Pelger-Huët neutrophils as “others” and then define “others” as Pelger-Huët neutrophils.

Neutrophil Hypersegmentation

Normal neutrophils contain three to five lobes that are separated by filaments. Hypersegmented neutrophils have more than five lobes and are most often associated with the megaloblastic anemias, where the neutrophil is also larger than normal (Figure 29-2). Hypersegmented neutrophils can also be seen in the myelodysplastic syndromes and represent a form of myeloid dysplasia. Much less frequently, hypersegmented neutrophils can be found in hereditary neutrophil hypersegmentation. In this disorder, patients are asymptomatic and have no signs of megaloblastic anemia.

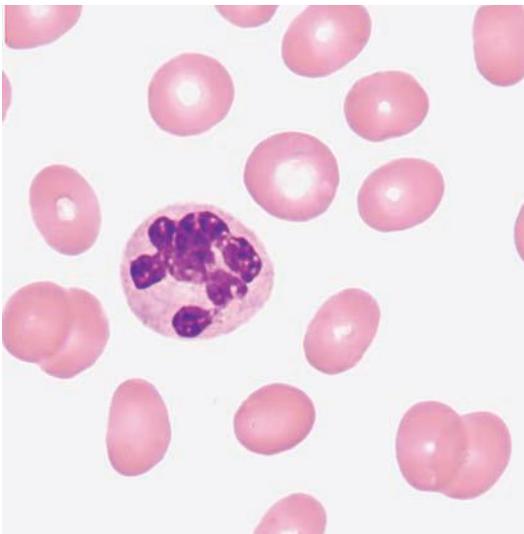


Figure 29-2 Hypersegmented neutrophil. (From Carr JH, Rodak BF: *Clinical hematology atlas*, ed 4, St. Louis, 2013, Saunders.)

Myelokathexis refers to a rare hereditary condition characterized by normal granulocyte production; however, there is impaired release into circulation that leads to neutropenia. Neutrophil morphology is also affected. Neutrophils appear hypermature. There may be hypersegmentation, hypercondensed chromatin, and pyknotic changes. Cytoplasmic vacuoles may also be observed.⁸ Myelokathexis is a component of an extremely rare inherited disorder, **WHIM**, a syndrome in which warts, neutropenia, hypogammaglobulinemia, infections, and myelokathexis are common findings.^{9,10} Mutations in *CXCR4* result in a hyperfunctional CXCR4 receptor and ligand binding, which impairs cellular homeostasis and trafficking, leading to neutropenia, lymphopenia, and hypogammaglobulinemia.^{11,12}

Alder-Reilly Anomaly

Alder-Reilly anomaly is transmitted as a recessive trait and is characterized by granulocytes with large, darkly staining metachromatic cytoplasmic granules composed primarily of partially digested mucopolysaccharides. The granules are referred to as Alder-Reilly bodies or Reilly bodies. The morphology may resemble heavy toxic granulation, which is discussed later (Figure 29-3). Neutrophilia, Döhle bodies, and left shift, which are usually associated with toxic granulation, are not seen in Alder-Reilly anomaly. Also, in some patients with Alder-Reilly anomaly, the granules are found in lymphocytes and monocytes, ruling out toxic granulation, which is exclusive for neutrophils. The basic defect is the incomplete degradation of mucopolysaccharides. Reilly bodies are most commonly associated with Hurler syndrome, Hunter syndrome, and Maroteaux-Lamy polydystrophic dwarfism.¹³ Leukocyte function is not affected in Alder-Reilly anomaly.

Chédiak-Higashi Syndrome

Chédiak-Higashi syndrome is a rare, fatal, autosomal recessive disease. In 2008, only 800 cases were reported worldwide.¹⁴

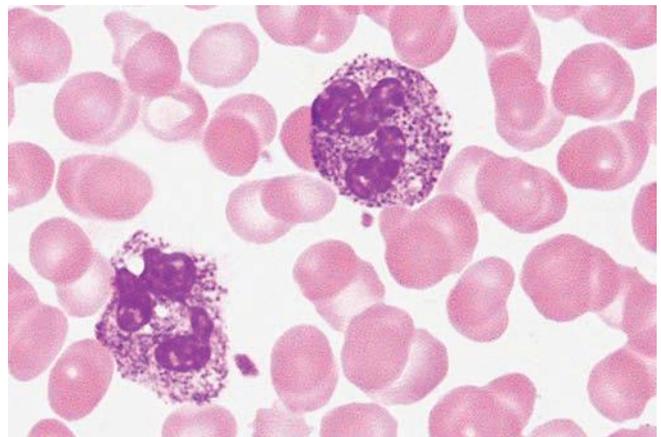


Figure 29-3 Two neutrophils from a patient with Alder-Reilly anomaly. Note the dark granules present in both cells. Such granules may also be seen in eosinophils and basophils. (Courtesy Dennis R. O'Malley, MD, US Labs, Irvine, CA.)

The disease is characterized by abnormal fusion of granules in most cells that contain granules throughout the body. The fused granules are large and mostly dysfunctional. Hematopoietic cells are affected, but disease manifestations can be found in hair, skin, adrenal and pituitary glands, and nerves. Hematologic findings in Chédiak-Higashi syndrome include giant lysosomal granules in granulocytes, monocytes, and lymphocytes (Figure 29-4). These fused granules result in leukocyte

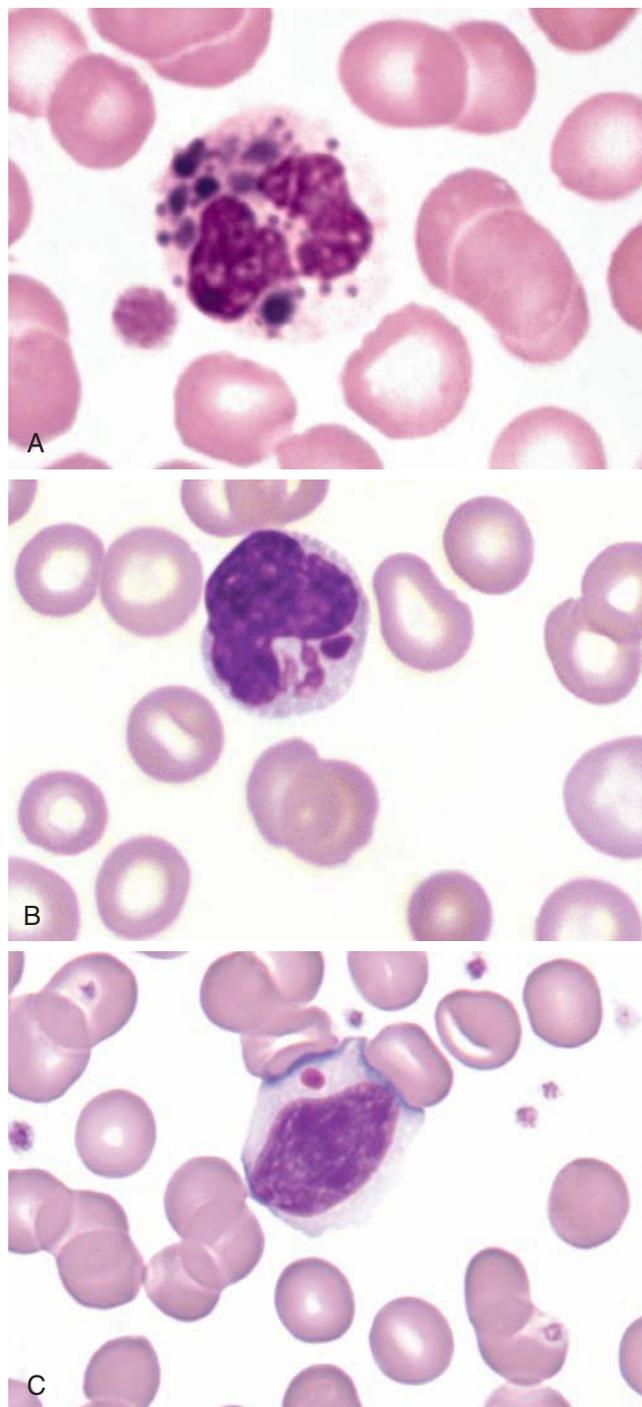


Figure 29-4 Three cells from a patient with Chédiak-Higashi syndrome. **A**, Neutrophil with large dark lysosomal granules. **B**, Monocyte with large azure granules. **C**, Lymphocyte with one large azure granule.

dysfunction and recurrent pyogenic infections. Patients often have bleeding issues due to abnormal dense granules in platelets.¹⁵ Chédiak-Higashi syndrome is associated with a mutation in the *CHS1* *LYST* gene on chromosome 1q42.1-2 that encodes for a protein involved in vesicle fusion or fission.¹⁵

May-Hegglin Anomaly

May-Hegglin anomaly is a rare, autosomal dominant platelet disorder characterized by variable thrombocytopenia, giant platelets, and large Döhle body-like inclusions in neutrophils, eosinophils, basophils, and monocytes (Figure 29-5). May-Hegglin anomaly is caused by a mutation in the *MYH9* gene on chromosome 22q12-13.¹⁶ There is disordered production of myosin heavy chain type IIA which affects megakaryocyte maturation and platelet fragmentation.¹⁶ The basophilic Döhle body-like leukocyte inclusions in May-Hegglin anomaly are composed of precipitated myosin heavy chains. Döhle bodies are composed of lamellar rows of rough endoplasmic reticulum. Clinically, the majority of individuals with May-Hegglin anomaly are asymptomatic, but a few have mild bleeding tendencies that are related to the degree of thrombocytopenia.

A summary of morphologic changes and clinical findings associated with the above disorders is shown in Box 29-1.

Normal Morphology with Functional Abnormalities

The majority of genetic functional leukocyte disorders, with the exception of some of the storage disorders, are not characterized by specific morphologic alterations in leukocytes. Box 29-2 outlines the causes and clinical findings seen in these disorders.

Chronic Granulomatous Disease

Chronic granulomatous disease is a rare inherited disorder caused by the decreased ability of phagocytes to produce superoxide and reactive oxygen species. Following phagocytosis of

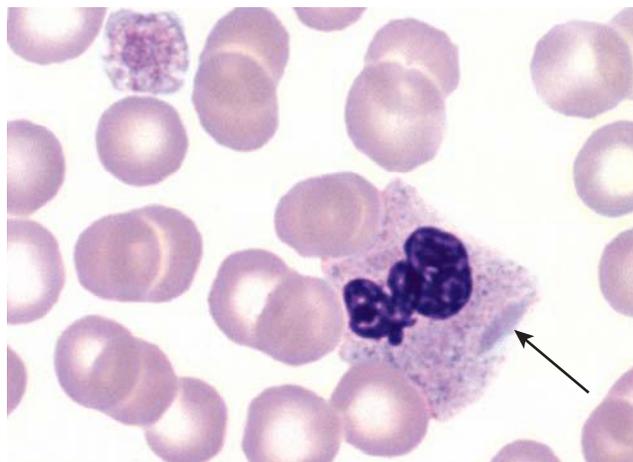


Figure 29-5 A neutrophil and a giant platelet from a patient with May-Hegglin anomaly. Note the large, elongated, bluish inclusion in the neutrophil cytoplasm.

BOX 29-1 Morphologic Abnormalities of Neutrophils with and without Functional Defects

Morphologic Abnormality	Morphologic Changes	Clinical Findings
Pelger-Huët anomaly	Decreased nuclear segmentation in neutrophils; sometimes also affects other WBCs	Asymptomatic
Pseudo-Pelger-Huët-anomaly	Decreased nuclear segmentation in neutrophils	Depends on underlying condition
Neutrophil hypersegmentation	>5 nuclear lobes in neutrophils	Depends on underlying cause
Alder-Reilly anomaly	Granulocytes contain large, darkly staining metachromatic cytoplasmic granules	Normal neutrophil function. Clinical findings, if present, are due to associated condition
Chédiak-Higashi disease	Giant lysosomal granules in granulocytes, monocytes, and lymphocytes	Leukocyte dysfunction and recurrent pyogenic infections; bleeding due to abnormal dense granules in platelets
May-Hegglin anomaly	Thrombocytopenia, giant platelets and large Döhle body–like inclusions in neutrophils, eosinophils, basophils, and monocytes	Usually asymptomatic; sometimes mild bleeding related to the degree of thrombocytopenia

Box 29-2 Normal Morphology of Neutrophils with Functional Abnormalities

Disorder	Cause(s)	Clinical Findings
Chronic granulomatous disease	Mutation(s) in <i>NADPH oxidase</i> genes leads to failure of neutrophil respiratory burst following phagocytosis of organism	Heterogeneous, however most experience recurrent bacterial and fungal infections; granulomas may obstruct organs (liver, spleen, others)
Leukocyte adhesion disorder – I	Mutation in gene(s) responsible for β_2 integrin subunits, leads to decreased or truncated β_2 integrin, needed for neutrophil adhesion to endothelial cells, recognition of bacteria, and outside-in signaling	Recurrent infections, neutrophilia, lymphadenopathy, splenomegaly, and skin lesions; variable severity and survival
Leukocyte adhesion disorder – II	Mutation in <i>SLC35C1</i> which codes for a fucose transporter involved in synthesis of selectin ligands. Results in decreased amount or function of selectin ligands and defective leukocyte recruitment	Physical growth retardation, coarse face, and/or other physical deformities; neurological defects, recurring infections, and absent blood group H antigen
Leukocyte adhesion disorder – III	Mutations in <i>Kindlin-3</i> and defective protein product Kindlin-3, needed for β integrin activation and leukocyte rolling. Failed response to external signals that would normally result in leukocyte activation.	Recurrent bacterial and fungal infections (less severe than LAD-I). Decreased platelet integrin GPIIb β 3, resulting in bleeding

microorganisms, there is no respiratory burst that normally results in the production of these antimicrobial agents. The basic defect is one or more mutations in genes responsible for proteins that make up a complex known as *NADPH oxidase* (NADPH is the reduced form of nicotinamide adenine dinucleotide phosphate). Under normal conditions, phagocytosis of foreign organism leads to phosphorylation and binding of cytosolic p47_{phos} and p67_{phos}.¹⁷ Primary granules containing antibacterial neutrophil elastase and cathepsin G and secondary granules containing the cytochrome complex gp91_{phox} and gp22_{phox} migrate to the phagolysosome. NADPH oxidase forms when p47_{phos} and p67_{phos} along with p40_{phox} and RAC2 combine with the cytochrome complex. Superoxide is generated in the phagolysosome when an electron from NADPH is added to oxygen. NADPH has additional regulatory functions in the generation of other antimicrobial agents. Most cases of chronic granulomatous disease are due to mutations in gp91_{phox} or p47. The majority of cases (approximately 60% to 65%)

are X-linked recessive, whereas 35% to 40% are autosomal recessive.¹⁸

The disease is heterogeneous, and survival is based on the type of mutation, which in turn determines the level of superoxide produced.¹⁷ Most patients experience bacterial and fungal infections of the lung, skin, lymph nodes, and liver. Macrophage-rich granulomas can be found in the liver, spleen, and other organs. These granulomas sometimes obstruct the intestines, urinary tract, and lungs. Advancements in treatment, in particular antifungal agents have greatly increased survival rates, where 90% of patients survive well into adulthood.¹⁹ In the nitroblue tetrazolium reduction test, normal neutrophils, when stimulated, reduce the yellow water-soluble nitroblue tetrazolium to a dark blue insoluble formazan. Neutrophils in chronic granulomatous disease cannot perform this reduction (**Figure 29-6**). The disease can also be diagnosed through flow cytometry, which uses a fluorescent probe, such as dihydrorhodamine-123,

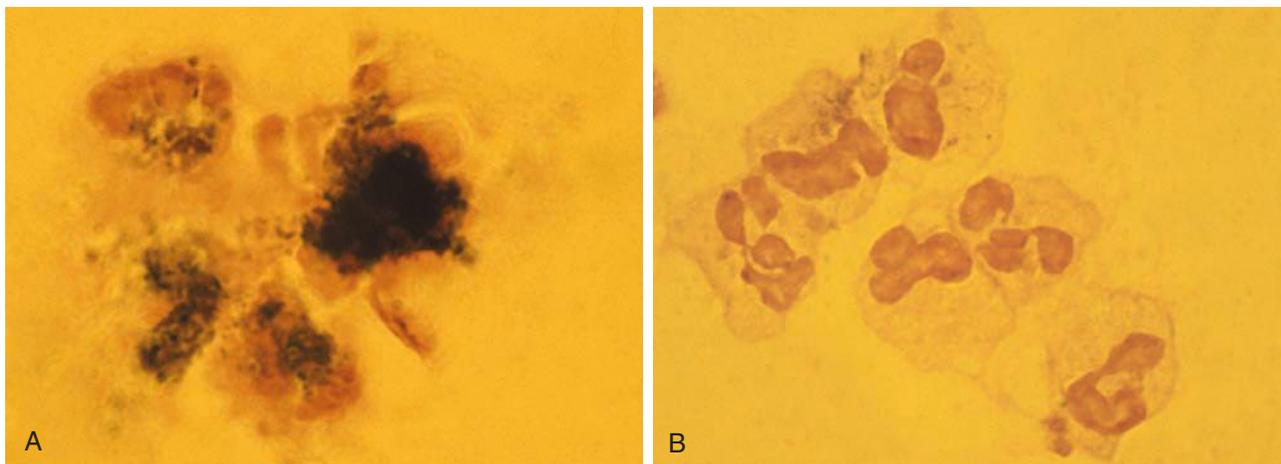


Figure 29-6 Two nitroblue tetrazolium preparations. **A**, Neutrophils from a normal control that have reduced the NBT to a dark formazan. **B**, Neutrophils from a patient with chronic granulomatous disease. (Courtesy Valerie Evans, University of Arizona Medical Center, Tucson, AZ.)

to measure intracellular production of reactive oxygen species.¹⁸

Leukocyte Adhesion Disorders

Recruitment of leukocytes to the site of inflammation involves capture of leukocytes from peripheral blood, followed by rolling along a vessel wall. This process is mediated through selectins, which interact with their ligands on the surface of leukocytes.²⁰ Ligand binding induces high-affinity binding of integrins with endothelial cell receptors. The cytoskeleton in leukocytes is reorganized, and cell spreading occurs, which ultimately leads to transmigration of the leukocyte out of the blood and into the tissues.

Leukocyte adhesion disorders (LADs) are rare autosomal recessive inherited disorders that result in the inability of neutrophils and monocytes to adhere to endothelial cells and to transmigrate from the blood to the tissues. The consequence is increased and potentially lethal bacterial infections. The basic defect is a mutation in the genes responsible for the formation of cell adhesion molecules (Chapter 12).

Leukocyte adhesion disorders have been subdivided into three subcategories. LAD I is caused by a mutation in exons 5 to 9 in the gene(s) responsible for β_2 integrin subunits, resulting in either a decreased or truncated form of the β_2 integrin,²¹ which is necessary for adhesion to endothelial cells, recognition of bacteria, and outside-in signaling.²² In addition to experiencing recurrent infections, patients with LAD I frequently have neutrophilia, lymphadenopathy, splenomegaly, and skin lesions. The clinical severity, including number of infections and survival, depends on the amount of the β_2 integrins produced.²³

LAD II is considerably rarer than LAD I and presents in a similar manner (recurrent infection and neutrophilia), but the leukocytes have normal β_2 integrins. There are molecular defects in *SLC35C1*, which codes for a fucose transporter that moves fucose from the endoplasmic reticulum to the Golgi region.²⁴ Fucose is needed for posttranslational fucosylation of glycoconjugates, which is required for synthesis of selectin

ligands.²⁵ Clinically, LAD II patients have growth retardation, a coarse face, and other physical deformities.²⁶ In LAD II the defective fucose transporter leads to an inability to produce functional selectin ligands and defective leukocyte recruitment, which leads to recurring infections. Other clinical findings related to defective fucose transport are absence of blood group H antigen, growth retardation, and neurological defects.²⁷

LAD III is a very rare autosomal recessive disease. In LAD III, leukocytes and platelets have normal expression of integrins, but there is failure in response to external signals that would normally result in leukocyte activation.²³ Mutations in *Kindlin-3* have recently been identified as the culprit.²⁸ Kindlin-3 protein along with talin are required for activation of β integrin and leukocyte rolling. Clinically LAD III patients experience a mild LAD I-like immunodeficiency with recurrent bacterial and fungal infections. In addition, in LAD III, there is decreased platelet integrin GPIIb β 3, resulting in bleeding similar to what is seen in Glanzmann thrombasthenia (Chapter 41).

Miscellaneous Granulocyte Disorders

Myeloperoxidase (MPO) deficiency is characterized by a deficiency in myeloperoxidase in the primary granules of neutrophils and lysosomes of monocyte. Myeloperoxidase normally stimulates production of hypochlorite and hypochlorous acid, which are oxidant agents that attack phagocytized microbes. The disorder is inherited in an autosomal dominant manner with a prevalence of approximately 1 in 2000 individuals.²⁹ The defect originates through mutation in the *MPO* gene on chromosome 17. Most patients do not experience problematic recurring infections because compensatory pathways are utilized for microbe killing that do not involve myeloperoxidase.²⁹ Acquired myeloperoxidase deficiency can present in association with hematologic neoplasms and lead poisoning.³⁰ In the hematology laboratory, MPO deficiency can be easily detected by the Siemens Advia analyzer, which uses myeloperoxidase to identify cells in the automated differential.

Monocyte/Macrophage Lysosomal Storage Diseases

Monocyte/macrophage lysosomal storage diseases can be subdivided into mucopolysaccharide (or glycosaminoglycan [GAG]) storage diseases and lipid storage diseases (Table 29-1). As a group, they represent inherited enzyme deficiencies or defects that result in flawed degradation of phagocytized material and buildup of the partially digested material within the phagocyte. All cells containing lysosomes can be affected, including T lymphocytes.³¹

The *mucopolysaccharidoses* (MPSs) are a family of inherited disorders of GAG degradation. Each MPS is caused by deficient activity of an enzyme necessary for the degradation of dermatan sulfate, heparan sulfate, keratan sulfate, and/or chondroitin sulfate. The partially degraded GAG builds up in the lysosomes and eventually results in physical abnormality and sometimes mental retardation. The MPSs have been subdivided according to which enzyme is defective, which GAG is being stored, and whether the symptoms are severe or attenuated (Table 29-1).³²

The peripheral blood of a patient with MPSs may appear relatively normal; however, metachromatic Reilly bodies may be seen in neutrophils, monocytes, and lymphocytes (Figure 29-7). Bone marrow may reveal macrophages with large amounts of metachromatic material. Diagnosis relies on assays for the specific enzymes involved. Treatment has consisted of enzyme replacement therapy or hematopoietic stem cell transplantation.³²

Lipid storage diseases are inherited disorders in which lipid catabolism is defective (Figure 29-8). Two of these disorders are characterized by macrophages with distinctive morphology and are discussed here.

Gaucher disease is the most common of the lysosomal lipid storage diseases. It is an autosomal recessive disorder caused by a defect or deficiency in the catabolic enzyme β -glucocerebrosidase (gene located at *1q21*), which is necessary for glycolipid metabolism. At least 1 in 17 Ashkenazi Jews

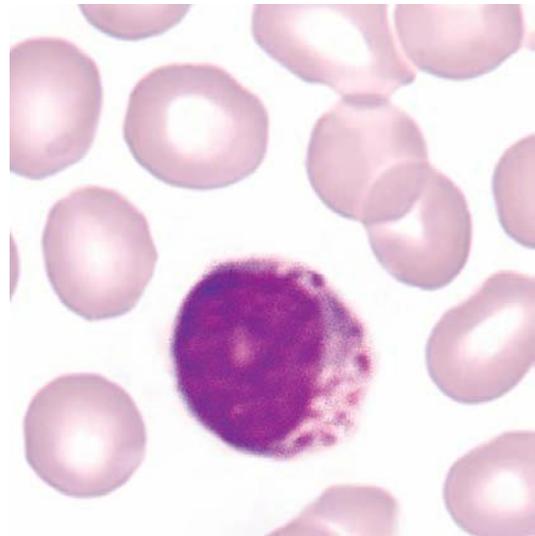


Figure 29-7 Lymphocyte on the blood film for a patient with a mucopolysaccharide storage disorder known as Hurler disease. Notice the dark cytoplasmic granules.

are carriers.³³ More than 300 genetic mutations have been reported,³⁴ and while some correlations have been found with specific mutations and disease severity and course, the majority of cases (phenotypes) cannot be predicted by genotype. In Gaucher disease there is an accumulation of unmetabolized substrate sphingolipid glucocerebroside in macrophages throughout the body, including osteoclasts in bone and microglia in the brain.

The clinical triad used in diagnosis is hepatomegaly, Gaucher cells in the bone marrow, and increase in serum phosphatase. Gaucher disease has been subdivided into three types based on clinical signs and symptoms (Table 29-2).³⁵ Neurologic symptoms play a key role in differentiating between the three subtypes. The phenotype is quite heterogeneous, with some patients being completely asymptomatic (seen in Type I),

TABLE 29-1 Variants of Monocyte/Macrophage Lysosomal Storage Disorders

Type	Name	Deficient Enzyme	Substance Stored
Mucopolysaccharidosis			
MPS I—severe	Hurler syndrome	α -L-iduronidase	Dermatan sulfate, heparan sulfate
MPS I—attenuated	Scheie syndrome	α -L-iduronidase	Dermatan sulfate, heparan sulfate
MPS II—severe	Hunter syndrome	Iduronate sulfatase	Dermatan sulfate, heparan sulfate
MPS III	Sanfilippo syndrome type A	Heparan N-sulfatase	Heparan sulfate
	Sanfilippo syndrome type B	α -N-acetylglucosaminidase	Heparan sulfate
	Sanfilippo syndrome type C	Acetyl-coenzyme A: α -glucosaminide N-acetyltransferase	Heparan sulfate
MPS IV	Morquio syndrome type A	Galactose-6-sulfatase	Keratan sulfate, chondroitin-6-sulfate
	Morquio syndrome type B	β -Galactosidase	Keratan sulfate
Lipid Storage Diseases			
	Gaucher disease	β -Glucocerebrosidase	Glucocerebroside
	Niemann-Pick disease	Sphingomyelinase	Sphingomyelin
	Fabry disease	α -Galactosidase	Ceramide trihexoside
	Tay-Sachs disease, Sandhoff disease	Hexosaminidase A	G _{M2} ganglioside

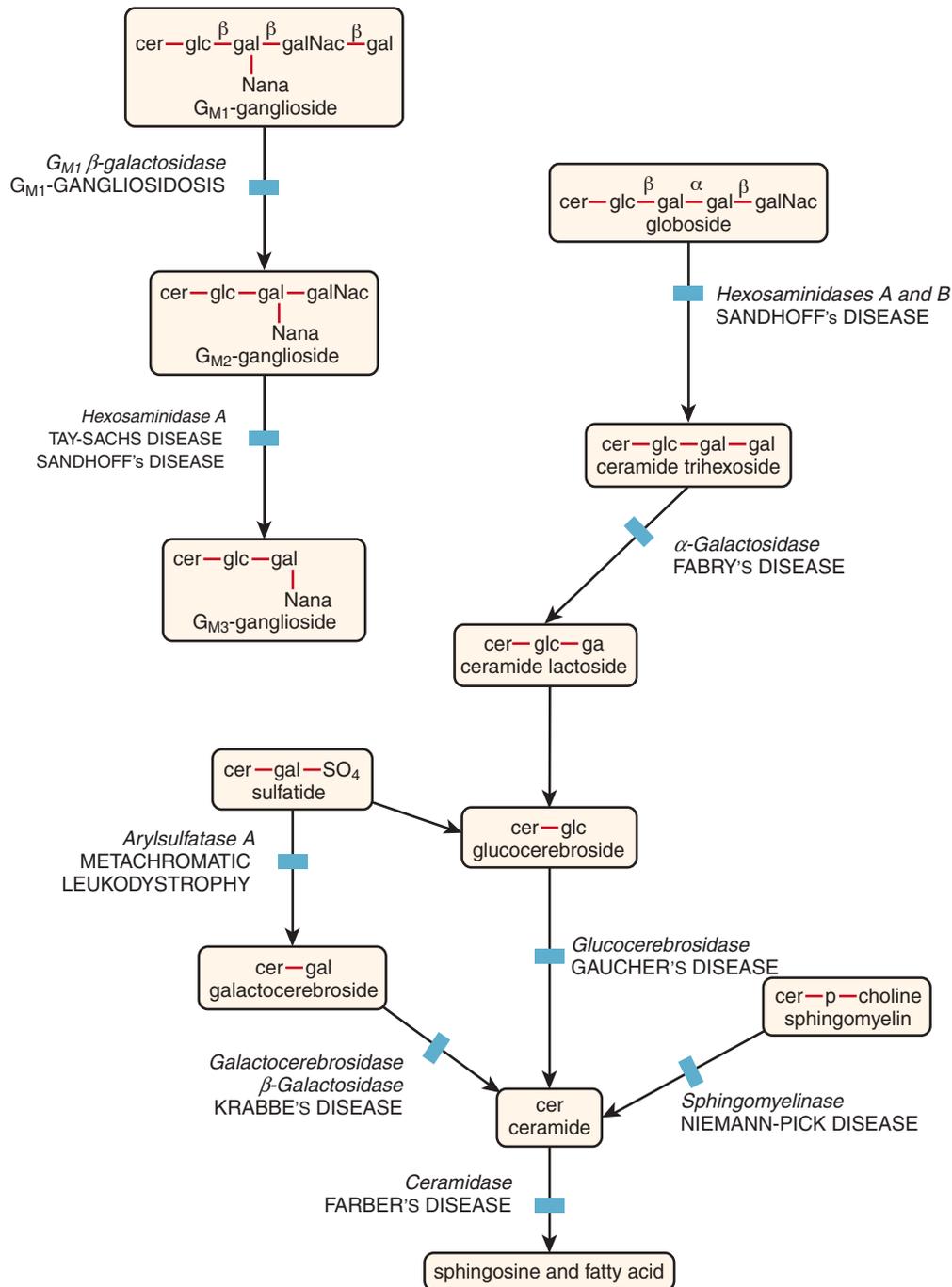


Figure 29-8 Pathways and diseases of sphingolipid metabolism. (From Orkin SH, Fisher DE, Look AT, et al: *Nathan and Oski's hematology of infancy and childhood*, ed 7, Philadelphia, 2009, Saunders.)

TABLE 29-2 Clinical Subtypes of Gaucher Disease

Clinical Features	Type I: Nonneuropathic	Type II: Acute Neuropathic	Type III: Subacute Neuropathic
Clinical onset	Childhood/adulthood	Infancy	Childhood
Hepatosplenomegaly	+	+	+
Skeletal abnormality	+	-	+
Neurodegeneration	-	+++	++
Death	Variable	By 2 years	Second to fourth decade
Ethnic predilection	Ashkenazi Jews	Panethnic	Swedes

NOTE: Absence and severity of features are indicated by - to +++.

while others experience a multitude of clinical problems. Clinical findings are mostly related to the patient's age and the degree of the enzyme deficiency.

Hematologic features include anemia and thrombocytopenia as a result of hypersplenism that is common in these patients. Bone marrow replacement by Gaucher cells may contribute to peripheral cytopenias. The bone marrow contains Gaucher cells, distinctive macrophages occurring individually or in clusters, that have an abundant fibrillar blue-gray cytoplasm with a striated or wrinkled appearance (sometimes described as onion skin-like) (Figure 29-9).³⁶ A useful test in terms of determining the level of glucocerebroside in storage is chitotriosidase.³⁷ This biomarker can be used in diagnosis and monitoring of the disease. The periodic acid-Schiff stain tests for mucopolysaccharides in Gaucher cells. Polymerase chain reaction is sometimes used in Ashkenazi Jews to screen for the most common mutations, but to confirm a diagnosis of Gaucher disease, gene sequencing is often needed. In all three forms of the disease, there is a fifteenfold increase for developing hematologic malignancies such as plasma cell neoplasm, chronic lymphocytic leukemia, lymphoma, and acute leukemia.³⁸

Treatment of Gaucher disease includes the use of enzyme replacement therapy with recombinant glucocerebrosidase.^{39,40} Agents are also available that reduce the amount of the substrate glucocerebroside. Stem cell transplantation offers the potential for cure, but safety is always a concern in allogeneic transplant, where the mortality rate associated with the procedure is high.⁴¹ Another treatment approach is the use of pharmacologic chaperones that are active site-specific competitive glucocerebroside inhibitors.⁴²

Pseudo-Gaucher cells can be found in the bone marrow in some patients with thalassemia,⁴³ chronic myelogenous leukemia,⁴⁴ and acute lymphoblastic leukemia.⁴⁵ In these diseases, pseudo-Gaucher cells form as a result of excessive cell turnover and overwhelming the glucocerebrosidase enzyme rather than a true decrease in the enzyme. Electron

microscopy can distinguish between the two cells because pseudo-Gaucher cells do not contain the tubular inclusions described in Gaucher cells.

Niemann-Pick disease is an autosomal recessive lipid storage disease that has three subtypes: A, B, and C. Types A and B are characterized by recessive mutations in the *SMPD1* gene, which leads to a deficiency in the lysosomal hydrolase enzyme acid sphingomyelinase (ASM) and a subsequent buildup of the substrate sphingomyelin in the liver, kidney, and lungs. In type A, the brain is also affected. In types A and B, Niemann-Pick cells are usually found in the bone marrow. These are macrophages with a foamy cytoplasm packed with lipid-filled lysosomes that appear as vacuoles after staining (Figure 29-10). Type A presents in infancy and is associated with a failure to thrive, hepatosplenomegaly, and a rapid neurodegenerative decline that results in death, usually by age 3 years. In type A, there is less than 5% of normal sphingomyelinase activity. Type B patients have approximately 10% to 20% normal enzyme activity,⁴⁶ and the disease presents later in life with a variable clinical course. These patients have little or no neurological symptoms, but many experience severe and progressive hepatosplenomegaly, heart disease, and pulmonary insufficiency.⁴⁷

In Niemann-Pick type C disease there is a decrease in cholesterol effluxing from the intracellular endosome/lysosome to the cytosol. In normal conditions this process is under control of two proteins: Niemann-Pick C I and Niemann-Pick C II. Mutations in these genes results in Niemann-Pick type C disease, where cholesterol, bisphosphate, and sphingolipids build up in lysosomal storage organelles of macrophages.⁴⁸ Patients with type C disease present with systemic, neurologic, and psychiatric symptoms.⁴⁹ The prognosis in type C is poor. Most patients die before the age of 25 years. The diagnosis can be confirmed through gene sequencing that identifies mutations

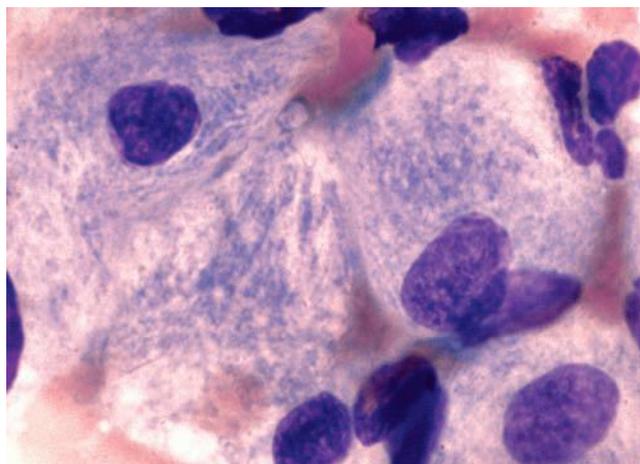


Figure 29-9 Characteristic macrophages with cytoplasmic striations found in the bone marrow of a patient with Gaucher disease. (From Carr JH, Rodak BF: *Clinical hematology atlas*, ed 4, St. Louis, 2013, Saunders.)

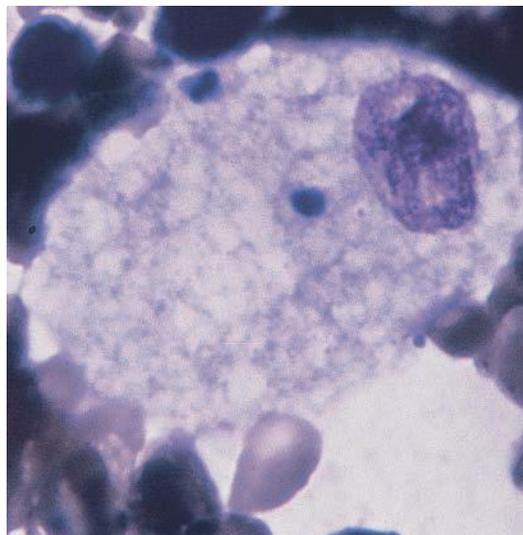


Figure 29-10 Niemann-Pick cell with eccentric nucleus and bubble-like pattern of storage deposit in the cytoplasm. (From Carr JH, Rodak BF: *Clinical hematology atlas*, ed 4, St. Louis, 2013, Saunders.)

in *NPC1* in 95% of cases. Five percent of patients will have mutations in *NPC2*.^{50,51}

Morphology, disease characteristics and associated laboratory tests for Gaucher and Niemann-Pick disease are highlighted in Box 29-3.

Genetic B and T Lymphocyte Abnormalities

Functional B and T lymphocyte abnormalities are genetic disorders that generally result in the decreased production of B cells, T cells, or both. They are all associated with an increased risk of infection and secondary malignancy.

T lymphocyte abnormality is best represented by a condition known as *DiGeorge syndrome*. This syndrome is characterized by the absence or underdevelopment of the thymus and thus markedly decreased numbers of T lymphocytes. It is associated with a microdeletion in chromosome band 22q11.2, which is the most common chromosome deletion and occurs in approximately 1 in 3500 births. Individuals with this deletion have a broad range of abnormalities, including defective parathyroid glands, cardiac abnormalities, abnormal facial development, neurologic disorders, and hypocalcemia. Less than 1% of patients with this deletion are athymic, a condition sometimes referred to as *complete DiGeorge syndrome*.⁵² Many of these patients are treated with thymus transplantation, with an approximately 75% survival rate.⁵³

Sex-linked agammaglobulinemia (XLA) is a B cell disease that is caused most frequently by a mutation in the gene encoding Bruton tyrosine kinase (BTK). Such mutations result in decreased production of BTK, which is important for B cell development, differentiation, and signaling. Without BTK, B lymphocytes fail to reach maturity and will die prematurely.^{54,55} Infants with XLA start to display symptoms after 1 to 2 months, once maternal antibodies have been cleared. Recurring bacterial infections ensue and can be life-threatening.

Combined B lymphocyte/T lymphocyte abnormalities include severe combined immunodeficiency (SCID) and Wiskott-Aldrich syndrome. SCID can be divided into two types: adenosine deaminase deficiency and X-linked SCID. Both result in depletions of T, B, and natural killer (NK) lymphocytes. Adenosine deaminase deficiency results in excess amounts of its natural substrates (adenosine and 2'-deoxyadenosine), which cause lymphocyte depletion through a variety of mechanisms.⁵⁶

X-linked SCID is the more common of the two and is caused by a mutation in the gene encoding the IL-2 receptor γ chain, which is shared by several interleukins. The mutation results in T cell lymphopenia, B cells that are dysfunctional, and a lack of NK cells.⁵⁷

Wiskott-Aldrich syndrome is also X-linked and is caused by a mutation in a gene that encodes a protein called *WASp*. The mutation results in low levels or absence of the protein, and affected individuals have immunodeficiency, eczema, and thrombocytopenia. Absence of *WASp* affects migration, adhesion, and activation of a variety of leukocytes, including T cells, B cells, and NK cells.⁵⁸

QUANTITATIVE ABNORMALITIES OF LEUKOCYTES

Neutrophils

The age-specific reference range for leukocyte subsets is listed in the cover of this book. An increase in neutrophils above $7.0 \times 10^9/L$ in adults and $8.5 \times 10^9/L$ in children is referred to as *neutrophilia*. The normal relative neutrophil count is approximately 50% to 70%; however, neutrophilia should always be evaluated using absolute values. The absolute neutrophil count (ANC) is determined by adding the number of segmented and band neutrophils. Some laboratories calculate

Box 29-3 Lipid Storage Disease Characteristics and Associated Laboratory Tests

Disease	Morphologic Changes	Clinical Findings	Laboratory Test
Gaucher	Macrophages with blue-gray cytoplasm appear striated or wrinkled (onion skin–like)	Variable depending on subtype: includes neurologic symptoms, hypersplenism, anemia, thrombocytopenia	Chitotriosidase: determines level of storage glucocerebrosidase Periodic-acid-Schiff: stains glucocerebrosidase in macrophages, polymerase chain reaction and gene sequencing: screens for associated genetic mutations
Niemann-Pick type A	Macrophages contain foamy cytoplasm packed with lipid-filled lysosomes that appear as vacuoles after staining	Failure to thrive in infancy, hepatosplenomegaly, rapid neurodegenerative decline that results in death, usually by age 3 years	Acid sphingomyelinase activity level distinguishes type A vs. B
Niemann-Pick type B	Same as above	Presents in first decade to adulthood, hepatosplenomegaly, heart disease, and pulmonary insufficiency, no neurological symptoms	Acid sphingomyelinase activity level distinguishes type A vs. B
Niemann-Pick type C	Same as above	Systemic, neurologic, and psychiatric symptoms, poor prognosis, most patients die before the age of 25 years	Gene sequencing to screen for <i>NPC1</i> mutations (95% of cases) or <i>NPC2</i> (5% of cases)

the ANC differently and include metamyelocytes, or metamyelocytes and myelocytes in the count. An increase in neutrophils can be related to several factors, including catecholamine-induced demargination or a shift in neutrophils from the marginal pool (cells normally adhering to vessel walls) to the circulating pool. This can occur from strenuous exercise, emotional stress, shock, burns, trauma, labor, or an increase in epinephrine. Neutrophilia also occurs in conditions that result in an increase in bone marrow production or in the shift of neutrophils from the bone marrow storage pool to the peripheral blood. The latter is almost always accompanied by a shift to the left (presence of immature neutrophils). Table 29-3 lists the major causes of neutrophilia.

The term *leukemoid reaction* refers to a reactive leukocytosis above $50 \times 10^9/L$ with neutrophilia and a marked left shift (presence of immature neutrophilic forms). Leukemoid reactions are mostly a result of acute and chronic infection, metabolic disease, inflammation, or response to a malignancy. Leukemoid reaction most often refers to neutrophils, but the increased count may be due to an increase in other types of leukocytes. A neutrophilic leukemoid reaction may be

confused with chronic myelogenous leukemia. There are several distinguishing features between the two that are listed in Box 29-4.

The term *leukoerythroblastic reaction* refers to the presence of immature neutrophils, nucleated red blood cells, and teardrop RBCs in the same sample. Leukoerythroblastic reactions are often accompanied by neutrophilia, but not always. Leukoerythroblastic reactions point to the possibility of a space-occupying lesion in the bone marrow. A space-occupying lesion can be a metastatic tumor, fibrosis, lymphoma, leukemia, or simply a marked increase in one of the normal marrow cells (e.g., erythroid hyperplasia seen in hemolytic anemia). A leukoerythroblastic reaction is also strongly associated with primary myelofibrosis.

Neutropenia refers to a decrease in the absolute neutrophil count (ANC) below $2.0 \times 10^9/L$ in white adults and $1.3 \times 10^9/L$ in black adults. The risk of infection increases as the ANC lowers, especially below $1.0 \times 10^9/L$. Severe neutropenia ($<0.5 \times 10^9/L$) further increases the risk. *Agranulocytosis* refers to neutrophil counts of less than $0.5 \times 10^9/L$. Some causes of neutropenia are an increased rate of

TABLE 29-3 Causes of Nonmalignant Neutrophilia and Neutropenia

Neutrophilia	Neutropenia	Neutrophilia	Neutropenia
<ul style="list-style-type: none"> Emotional Strenuous exercise Trauma/injury Pregnancy: labor and delivery Postsurgery Acute hemorrhage Infections: bacterial, some viral Burns Surgery Myocardial infarction Pancreatitis Vasculitis Colitis Autoimmune disease Acute hemorrhage/hemolysis Steroids Lithium Colony-stimulating factors (G-CSF) Smoking Chronic blood loss Metabolic ketoacidosis 	<ul style="list-style-type: none"> Drugs <ul style="list-style-type: none"> Analgesic/Anti-inflammatory: acetaminophen, ibuprofen, indomethacin Antibiotics: cephalosporins, chloramphenicol, clindamycin, gentamicin, penicillin, vancomycin, tetracycline Anticonvulsants: carbamazepine, mephenytoin, phenytoin Antidepressants: amitriptyline, amoxapine, doxepin Antihistamines: cimetidine, ranitidine Antimalarials: chloroquine, dapsone, quinine Cardiovascular: methyl dopa, propranolol, captopril Diuretics: chlorothiazide, hydrochlorothiazide Antianxiety/hypnotics: benzodiazepines, meprobamate Hypoglycemics: chlorpropamide, tolbutamide Phenothiazides: chlorpromazine, phenothiazines Others: allopurinol, clozapine, levamisole Radiation Toxins Immune mediated <ul style="list-style-type: none"> Alloimmune neonatal neutropenia Autoimmune neutropenia Lupus erythematosus Rheumatoid arthritis 	<ul style="list-style-type: none"> Uremia Eclampsia Malignancy Leukocyte adhesion deficiency Familial cold urticaria Hereditary neutrophilia 	<ul style="list-style-type: none"> Sjögren syndrome Felty syndrome Overwhelming infections Splenomegaly Hemodialysis Decreased or ineffective hematopoiesis Copper deficiency Alcoholism Babies born from hypertensive mothers Constitutional Shwachman-Diamond syndrome Severe congenital neutropenia Cyclic neutropenia Fanconi anemia Dyskeratosis congenita Common variable immunodeficiency syndrome Hyper-IgM syndrome X-linked agammaglobulinemia Reticular dysgenesis Glycogen storage disease, type Ib Chediak-Higashi syndrome Griselli syndrome, type 2 Hermansky-Pudlak syndrome, type 2 Myelokathexis/WHIM syndrome Barth syndrome Cohen syndrome

Adapted from Kaushansky K: *Williams hematology*, ed 8, New York, 2010, McGraw-Hill, pp 951-950; and Foucar K: *Bone marrow pathology*, ed 3, Chicago, 2010, ASCP Press, p 209.

BOX 29-4 Distinguishing between Chronic Myelogenous Leukemia and Leukemoid Reaction

Chronic Myelogenous Leukemia (Malignant)

Increases in all granulocytes, including eosinophils and basophils.

Occasional blast may be seen.

Dyspoietic morphology such as mixed eosinophil/basophil granulation and pseudo-Pelger-Huët cells may be encountered.

Involvement of platelets (may be increased), including giant, hypogranular, and/or bizarre forms.

Leukocyte alkaline phosphatase score is markedly decreased.

Leukemoid Reaction (Reactive)

Increase in neutrophils, including immature forms. Blasts are only rarely seen.

Reactive morphologic changes, including toxic granulation, Döhle bodies, and, less commonly, cytoplasmic vacuolization, are present.

Normal platelet morphology and number.

Increased leukocyte alkaline phosphatase score.

removal/destruction of neutrophils from the blood; fewer neutrophils being released from the bone marrow to the blood as a result of decreased production or ineffective hematopoiesis, where neutrophils are present in the bone marrow but are not released into the blood because they are defective; changes in ratio of circulating versus marginal pool of neutrophils; or a combination of the above.

Neutropenia can also be classified as inherited or acquired. [Table 29-3](#) lists the causes of neutropenia. Acquired causes of neutropenia are much more common than inherited causes. Immune-mediated neutropenia is caused by antibody binding to neutrophil antigens. In **alloimmune neonatal neutropenia**, maternal IgG crosses the placenta and binds to neutrophil-specific antigens inherited from the father, such as Fc γ RIIIb, NB1, or HLA.^{59,60} Alloimmune neonatal neutropenia occurs in approximately 1 in 2000 births. The severity of the neutropenia varies. Neutrophil counts rise after a few months, consistent with the half-life of the maternal antibody. **Autoimmune neutropenia in children** is a primary illness, with moderate to severe neutropenia developing as a result of antibodies to HNA-1. The disease tends to be self-limiting, with resolution of neutrophil counts after 7 to 24 months.⁶¹ **Secondary autoimmune neutropenia** is associated with autoimmune disorders such as rheumatoid arthritis and associated Felty syndrome, systemic lupus erythematosus, and Sjogren syndrome. In addition to antineutrophil antibodies, other factors may also induce neutropenia in secondary autoimmune neutropenia, including immune complex deposition, granulopoiesis-inhibiting cytokines, and splenomegaly. Over 100 drugs in use today are associated with neutropenia. [Table 29-3](#) contains a partial listing. The annual rate of occurrence is 3 to 12 cases per

million.^{62,63} The mechanism of drug-induced neutropenia may be related to a dose-dependent toxicity on cell replication in hematopoiesis. Another cause may be immunologic and occurs when a particular drug is given subsequent to the initial exposure that resulted in antibody formation.⁶⁴

Neutropenia may also result from infection, such as viral infection of hematopoietic progenitor cells, suppression of hematopoiesis by inflammatory cytokines, and increased usage of neutrophils due to overwhelming infection.

Intrinsic (constitutional/congenital) neutropenias are a relatively rare group of inherited disorders that usually present at birth. They are due to either decreased production from marrow hypoplasia or proliferation defect. Clinical presentation can be quite heterogeneous, but bacterial infections are the biggest risk. These infections can be life-threatening and/or diminish quality of life. However, antibiotic prophylaxis and therapy and the use of colony-stimulating factors such as G-CSF have lowered this risk and improved the quality of life for the majority of these patients.⁶⁵ All of the congenital neutropenias have an increased risk for developing secondary hematologic neoplasms, and G-CSF has been linked with an increased risk for secondary leukemia.^{66,67} The intrinsic causes of neutropenia are listed in [Table 29-3](#).

Shwachman-Diamond syndrome, or Shwachman-Bodian-Diamond syndrome, is an autosomal recessive disorder characterized by marrow failure, pancreatic insufficiency, and skeletal abnormalities. Intermittent neutropenia that fluctuates from severely low to near normal is the most common hematologic finding, affecting 88% to 100% of patients.⁶⁸ Mild normocytic to macrocytic normochromic anemia and reticulocytopenia is seen in approximately 80% of patients.^{69,70} Thrombocytopenia occurs in 24% to 88% of patients.^{71,72} Dysplastic changes involving all three granulocyte lineages are not uncommon. There is an increased risk for transformation to myelodysplasia and acute myeloid leukemia, which for many patients is a terminal event.

Congenital severe neutropenia consists of Kostmann syndrome and related diseases. Kostmann syndrome, or infantile genetic agranulocytosis, is an autosomal recessive disease characterized by severe neutropenia (often $<0.2 \times 10^9/L$) that presents shortly after birth and bone marrow granulocyte hypoplasia with maturation arrest at the promyelocyte stage. In cyclic neutropenia, approximately 50% of patients have mutations in *ELANE/ELA2*, the gene that codes for neutrophil elastase.⁷³ Patients with cyclic neutropenia have periods of severe neutropenia every 21 days, during which time there is increased risk for fevers, bacterial infections, mouth ulcers, and sometimes gangrene, bacteremia, and septic shock. Administration of G-CSF has greatly reduced these neutropenia-associated events.⁷⁴

Chronic idiopathic neutropenia in adults predominantly affects women 18 to 35 years of age. The bone marrow is quite variable between patients but generally shows more immature neutrophils than mature neutrophils, suggesting that cells are lost during maturation. Clinical severity is related to the degree of neutropenia. G-CSF has been shown to be a very useful treatment in these patients.

Fanconi anemia is a rare autosomal recessive or X-linked inherited disease characterized by variable degrees of bone marrow failure, peripheral cytopenias, and an increased risk for hematologic malignancies and other cancers.⁷⁵ Chapter 22 contains more information about Fanconi anemia.

Dyskeratosis congenita is a sex-linked recessive, autosomal dominant or autosomal recessively inherited disorder with a heterogeneous presentation.⁷⁶ In the classic form of the disease, patients have mucocutaneous abnormalities, abnormal skin pigmentation, nail dystrophy, and leukoplakia. Most patients also have bone marrow failure and increased risk for malignancy. Chapter 22 has more information about dyskeratosis congenita.

Eosinophils

Several factors influence the number of eosinophils in circulation: bone marrow proliferation rate and release into the bloodstream, movement from the blood into the extravascular tissues, and cell survival/destruction once the eosinophils have moved into the tissues. *Eosinophilia* is defined as an absolute eosinophil count above $0.4 \times 10^9/L$. Nonmalignant causes of eosinophilia are generally a result of cytokine stimulation, especially from interleukin-3 and interleukin-5 (IL3 and IL5).^{77,78} Most causes of eosinophilia can be divided into two broad categories, depending on geography. In underdeveloped areas of the world, increased peripheral blood eosinophils are seen in patients with parasite infestation, especially helminthes and protozoa. A major function of eosinophils is degranulation, where substances are released that damage an offending organism (i.e., parasites) or target cell.⁷⁹ In developed countries eosinophilia is most often associated with allergic conditions, including asthma, hay fever, urticarial, and atopic dermatitis.^{80,81} Eosinophilia is also seen in scarlet fever, HIV, fungal infections, autoimmune disorders, and hypersensitivity to antibiotics and anti-seizure medications. In addition, abnormalities in cytokine regulation and expression in some neoplasms result in a reactive eosinophilia. For example, reactive eosinophilia is seen in acute lymphoblastic leukemia, subtype t(5;14).⁸² In some cancers, eosinophils are able to penetrate solid tumors, allowing tumoricidal cytokines to bring about tumor necrosis.⁸³ If an individual is found to have eosinophilia ($>1.5 \times 10^9/L$) lasting more than 6 months without an identifiable cause, the diagnosis is most likely *hypereosinophilic syndrome*, or HES.⁸⁴ HES is considered to be a myeloproliferative neoplasm and will therefore not be discussed further in this chapter.

Eosinopenia is defined as an absolute eosinophil count of less than $0.09 \times 10^9/L$ and can be difficult to detect because the normal eosinophil reference range is very low. Eosinopenia is most often associated with conditions that result in marrow hypoplasia, specifically involving leukocytes. Another common cause of decreased eosinophils is infection or inflammation that is accompanied by neutrophilia. Eosinophils move into the tissues under these circumstances, and marrow release of eosinophils may be inhibited. Absolute eosinopenia has also been reported in autoimmune

disorders, steroid therapy, stress, sepsis, and acute inflammatory states.^{85,86}

Basophils

Basophilia is defined as an absolute basophil count greater than $0.15 \times 10^9/L$. The most common cause of basophilia is the presence of a malignant myeloproliferative neoplasm such as chronic myelogenous leukemia, which is covered in Chapter 33. Nonmalignant causes of basophilia are rare and include allergic rhinitis, hypersensitivity to drugs or food, chronic infections, hypothyroidism, chronic inflammatory conditions, radiation therapy, and bee stings.^{87,88}

Monocytes

Monocytosis is defined as an absolute monocyte count greater than $1.0 \times 10^9/L$ in adults and greater than $3.5 \times 10^9/L$ in neonates. Monocytosis is associated with a wide range of nonmalignant conditions because of their participation in acute and chronic inflammation and infections, immunologic conditions, hypersensitivity reactions, and tissue repair. Monocytosis is frequently the first sign of recovery from acute overwhelming infection or severe neutropenia (most commonly after cancer chemotherapy). Monocytosis in these conditions is considered a positive sign of recovery. Monocytosis when due to administration of G-CSF or GM-CSF may be accompanied by reactive changes in monocyte morphology. Monocytosis is associated with a number of neutropenic disorders. In cyclic neutropenia, monocytosis occurs inversely with neutropenia in the 21-day cycle. A listing of the conditions associated with nonmalignant monocytosis is provided in [Table 29-4](#).⁸⁹⁻⁹⁷

Monocytopenia, defined as an absolute monocyte count of less than $0.2 \times 10^9/L$, is very rare in conditions that do not also involve cytopenias of other lineage(s), such as aplastic anemia or chemotherapy-induced cytopenias. However, absolute monocytopenia has been found in patients receiving steroid therapy⁹⁸ or hemodialysis, or in sepsis.⁹⁹ Viral infections, especially those due to the Epstein-Barr virus (EBV), can cause monocytopenia ([Table 29-4](#)).¹⁰⁰

Lymphocytes

The definition of *lymphocytosis* varies with the age of the individual. Children older than 2 weeks and younger than 8 to 10 years normally have higher absolute lymphocyte counts than adults. Lymphocytosis in young children is defined as an absolute lymphocyte count greater than $10.0 \times 10^9/L$, whereas in adults it is defined as a count greater than $4.5 \times 10^9/L$. As can be seen from the tables inside the front cover of this book, newborn infants have lymphocyte counts very similar to those of adults. The reference range for relative lymphocytes is approximately 20% to 40%. This number, however, should not be used to define lymphocytosis. Blood smear review criteria should be based on the absolute numbers rather than the relative percentage of lymphocytes present.

Lymphocytoses can be subdivided into those with and those without reactive morphologic alterations. See [Table 29-5](#) for a listing of disorders that result in benign lymphocytosis.

TABLE 29-4 Reactive Causes of Monocytosis

Monocytosis
Infection
• Tuberculosis
• Viral
• Malaria
• Brucellosis
• Leishmaniasis
• Fungal
• Subacute bacterial endocarditis
• Syphilis
• Protozoal
Recovery from acute infection
Recovery from neutropenia
Immunologic/Autoimmune
• Systemic lupus erythematosus
• Rheumatoid arthritis
• Autoimmune neutropenia
• Inflammatory bowel disease
• Myositis
• Sarcoidosis
Hematologic
• Acute/chronic neutropenia
• Cyclic neutropenia
• Wiskott-Aldrich syndrome
• Drug-induced neutropenia
• Hemolysis
• Immune thrombocytopenia
Drugs
• Colony-stimulating factors
• Olanzapine
• Carbamazepine
• Phenytoin
• Phenobarbital
• Valproic acid
Cancer
• Carcinoma
• Sarcoma
• Plasma cell dyscrasias
• Lymphoma
Stress
• Trauma
• Myocardial infarction
• Intense exercise
Splenectomy
Gastrointestinal disease
• Alcoholic liver disease
• Sprue

Adapted from Foucar K: *Bone marrow pathology*, ed 3, Chicago, 2010, ASCP Press, p 199.

The definition of *lymphocytopenia* is age-dependent. Lymphocytopenia in young children is defined as an absolute lymphocyte count below $2.0 \times 10^9/L$, whereas in adults it is defined as a count below $1.0 \times 10^9/L$. Nonmalignant causes of lymphocytopenia can be subdivided into inherited and acquired and are listed in [Table 29-5](#).

TABLE 29-5 Causes of Nonmalignant Lymphocytosis and Lymphocytopenia

Lymphocytosis	Lymphocytopenia
Reactive Morphology	Inherited
Infection	Congenital immunodeficiency diseases
• Infectious mononucleosis	• Severe combined immunodeficiency disease
• Cytomegalovirus Infection	• Common variable immune deficiency
• Hepatitis	• Ataxia-telangiectasia
• Acute HIV infection	• Wiskott-Aldrich syndrome
• Adenovirus	• Others
• Chickenpox	
• Herpes	
• Influenza	
• Paramyxovirus (mumps)	
• Rubella (measles)	
• Roseola	
• Mumps	
• β -Hemolytic streptococci	
• Brucellosis	
• Paratyphoid fever	
• Toxoplasmosis	
• Typhoid fever	
• Listeria	
• Mycoplasma	
• Syphilis	
Miscellaneous	Acquired
• Idiosyncratic drug reactions	Aplastic anemia
• Postvaccination	Infections
• Sudden onset of stress from myocardial infarction	• Acquired immunodeficiency syndrome
• Allergic reaction	• Severe acute respiratory syndrome
• Hyperthyroidism	• West Nile
• Malnutrition	• Hepatitis
	• Influenza
	• Herpes
	• Measles
	• Tuberculosis
	• Typhoid fever
	• Pneumonia
	• Rickettsiosis
	• Ehrlichiosis
	• Sepsis
	• Malaria
	iatrogenic
	• Immunosuppressive agents
	• Stevens-Johnson syndrome
	• Chemotherapy
	• Radiation
	• Platelet or stem cell apheresis collection
	• Major surgery
	Systemic disease
	• Autoimmune diseases
	• Hodgkin lymphoma
	• Carcinoma
	• Primary myelofibrosis
	• Protein-losing enteropathy
	• Renal failure
	Nutritional/dietary
	• Ethanol abuse
	• Zinc deficiency

Adapted from Kaushansky K: *Williams hematology*, ed 8, New York, 2010, McGraw-Hill, pp. 1141-1151; and Foucar K: *Bone marrow pathology*, ed 3, Chicago, 2010, p 450.

QUALITATIVE (MORPHOLOGIC) CHANGES

Neutrophils

Neutrophil reaction to infection, inflammation, stress, or administration of recombinant colony-stimulating factor (CSF) therapy may affect the number and types of circulating neutrophils (left shift), induce morphologic change, or both. While these changes may be considered “abnormal,” they usually reflect a normal, reactive response. Depending on the severity of the infection, inflammation, or dose/reaction to CSF, the left shift can range from mild (an increase in band neutrophils and metamyelocytes) to moderate (metamyelocytes, myelocytes, and an occasional promyelocyte) to marked (myelocytes, promyelocytes, and an occasional blast form).

Reactive morphologic changes in neutrophils include toxic granulation, Döhle bodies, cytoplasmic vacuoles, hypersegmentation, and pyknosis. *Toxic granulation* of neutrophils appears as dark, blue-black granules in the cytoplasm of neutrophils: segmented, bands, and metamyelocytes. Toxic granules are peroxidase positive and reflect an increase in acid mucosubstance within primary, azurophilic granules of neutrophils.¹⁰¹ The result is a lowered pH in phagolysosomes that enhances microbial killing.¹⁰¹ There is a positive correlation between levels of C-reactive protein (acute phase protein) and the percentage of neutrophils with toxic granulation;¹⁰² therefore, the intensity of toxic granulation is a general measure of the degree of inflammation.¹⁰³ In addition, toxic granulation can be seen in various infections as well as in patients who have received CSF. Toxic granulation, especially when intense, can mimic the granulation found in the mucopolysaccharidoses and Alder-Reilly anomaly. One helpful defining characteristic of toxic granulation is that in most patients not all neutrophils are equally affected (Figure 29-11). Box 29-5A highlights reactive neutrophil morphologic changes and associated conditions.

Döhle bodies are cytoplasmic inclusions consisting of remnants of ribosomal ribonucleic acid (RNA) arranged in parallel rows.¹⁰⁴ Döhle bodies are typically found in band and

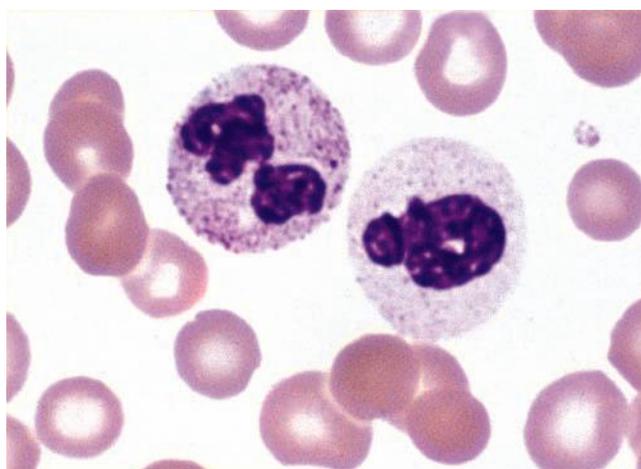


Figure 29-11 Toxic granulation. Note that one neutrophil contains toxic granulation and the other does not. Also note that the toxic granules are clustered in some areas of the cytoplasm. Both of these findings help in distinguishing toxic granulation from poor staining or from the dark granules seen in Alder-Reilly anomaly.

segmented neutrophils (Figure 29-12) and often in cells containing toxic granulation. They appear as intracytoplasmic, pale blue round or elongated bodies between 1 and 5 μm in diameter. They are usually located in close apposition to cellular membranes. A delay in preparing the blood after collection in EDTA tube may affect Döhle body appearance in that they are more gray than blue and in some cases may not be visible. Döhle bodies are relatively nonspecific. Their presence has been associated with a wide range of conditions, including bacterial infections, sepsis, and normal pregnancy.^{104,105}

Cytoplasmic vacuolation of neutrophils is seen less often than toxic granules and Döhle bodies. Vacuoles generally reflect phagocytosis, either of self (autophagocytosis) or of extracellular material. Autophagocytic vacuoles tend to be small (approximately 2 μm) and distributed throughout the cytoplasm. In addition, autophagocytosis can be induced by drugs such as sulfonamides and chloroquine,¹⁰⁰ storage in EDTA (artefactual) for more than 2 hours, autoantibodies,¹⁰⁶ acute alcoholism,¹⁰⁷ and exposure to high doses of radiation.¹⁰⁸ Phagocytic vacuoles caused by either bacteria or fungi are often seen in septic patients. Phagocytic vacuoles are large (up to 6 μm) and are frequently accompanied by toxic granulation (Figure 29-13). When vacuoles are seen, especially when not accompanied by toxic granulation, Döhle bodies, or both, artefactual causes should be suspected. The blood collection time should be compared with when the smear was made. As stated above, this time should not exceed 2 hours.

When phagocytic vacuoles are seen, a careful examination should be made for bacteria or yeast within the vacuoles. Cases of ehrlichiosis and anaplasmosis have been increasing in the United States over the past decade.¹⁰⁹ *Ehrlichia* and *Anaplasma* are small, obligate, intracellular bacteria transmitted by ticks to humans and other vertebrate hosts. These organisms grow as a cluster (morula) in neutrophils (*A. phagocytophilum* and *E. ewingii*) (Figure 29-14, A) and in monocytes (*E. chaffeensis*) (Figure 29-14, B). Leukopenia, thrombocytopenia, and elevated liver enzymes are common laboratory findings, and anemia may develop in about half the cases of human monocytic ehrlichiosis.¹¹⁰ Intracellular aggregates in neutrophils or monocytes may occasionally be detected in the first week of infection on a Wright-Giemsa-stained peripheral blood film or a buffy coat preparation. Immunofluorescent antibody titers or polymerase chain reaction testing may help to confirm the diagnosis.¹¹⁰ Early diagnosis is essential because antibiotic treatment with doxycycline is very effective and can prevent serious complications.

Pyknotic nuclei in neutrophils generally indicate imminent cell death. In a pyknotic nucleus, nuclear water has been lost and the chromatin becomes very dense and dark; however, filaments can still be seen between segments.^{111,112} Pyknotic nuclei should not be confused with necrotic nuclei found in dead cells. Necrotic nuclei are rounded fragments of nucleus with no filaments and no chromatin pattern (Figure 29-15).

Degranulation is a common finding in activated neutrophils and eosinophils (Figure 29-16). Both primary and secondary granules are emptied into phagosomes, and secondary granules are also secreted into the extracellular space.¹¹³ In vitro degranulation in eosinophils often occurs when cellular membranes are

Box 29-5A Reactive Morphologic Changes in Neutrophils

Reactive Change	Morphology	Associated with
Toxic granulation	Dark, blue-black cytoplasmic granules	Inflammation, infection, administration of granulocyte colony stimulation factor (G-CSF)
Dohle bodies	Intracytoplasmic pale blue round or elongated bodies between 1 and 5 μm in diameter, usually adjacent to cellular membranes.	Nonspecific finding, or associated with bacterial infections, sepsis, and pregnancy
Cytoplasmic vacuolization of neutrophils	Small to large circular clear areas in cytoplasm, rarely may contain organism	Septicemia or other infection; autophagocytosis secondary to drug ingestion, acute alcoholism, or storage artifact; vacuoles are sometimes seen in conjunction with toxic granulation.

disrupted during the process of making the blood film. Eosinophils are fragile.

Cytoplasmic swelling may be caused by actual osmotic swelling of the cytoplasm or by increased adhesion to the glass slide by stimulated neutrophils. Regardless of the cause, the result is a variation in neutrophil size or neutrophil anisocytosis (Figure 29-17).

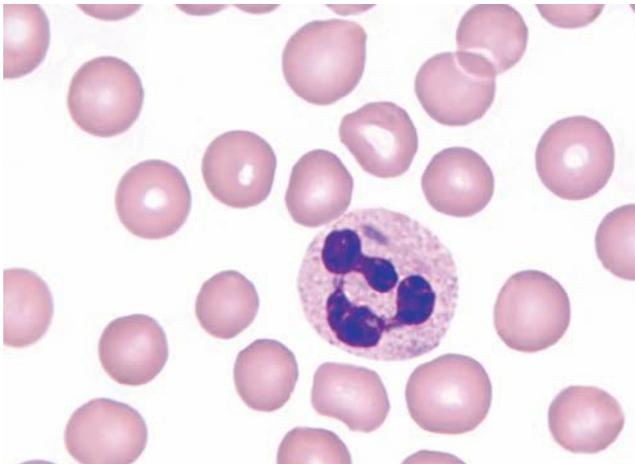


Figure 29-12 Neutrophil containing a bluish cytoplasmic inclusion known as a Döhle body.

Monocytes

Occasional immature monocytes may be seen in the peripheral blood in response to infection or inflammation, but this is not as common as a neutrophilic left shift (Figure 29-18). Reactive changes may be seen in monocytes in infections, during recovery from bone marrow aplasia, and after GM-CSF administration. The nucleus can become thin and band-like in areas and may appear to be segmenting (Figure 29-19). Reactive changes also include increased cytoplasmic volume, increased numbers of cytoplasmic granules, and evidence of phagocytic activity

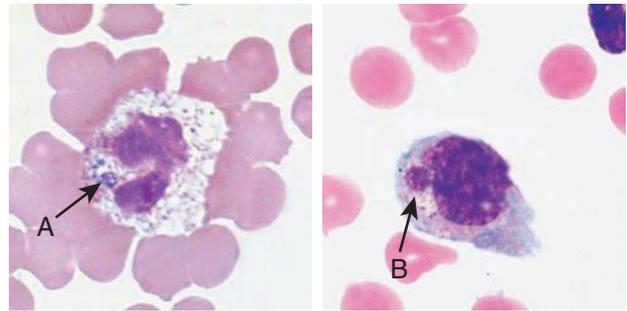


Figure 29-14 A, *Anaplasma phagocytophilum* in a neutrophil. B, *Ehrlichia chaffeensis* in a monocytic cell. (Courtesy J. Stephen Dumler, MD, Division of Medical Microbiology, The Johns Hopkins Medical Institution, Baltimore, MD.)

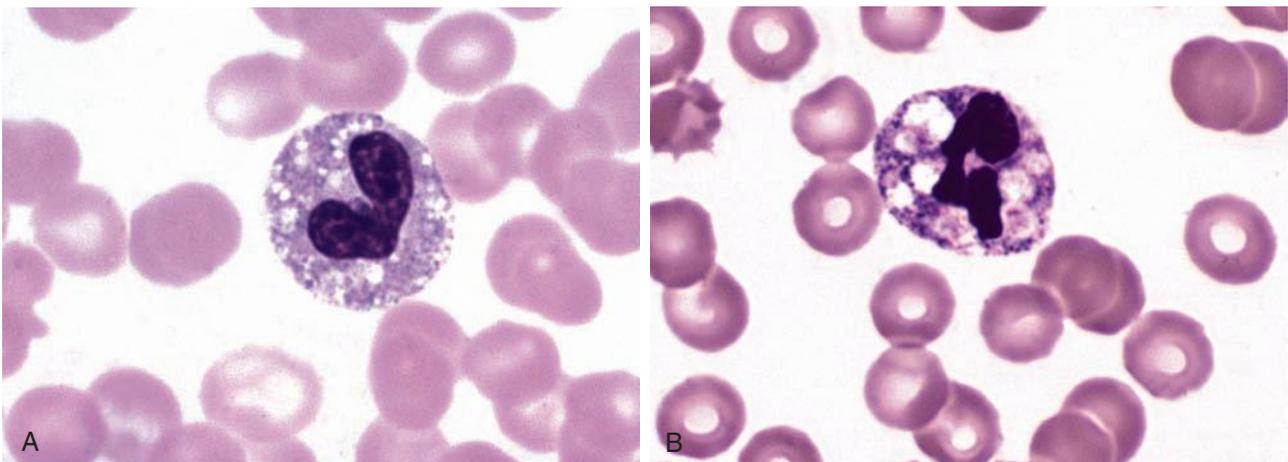


Figure 29-13 Cytoplasmic vacuoles. A, Band neutrophil with autophagocytic vacuoles. Note their small size. B, Neutrophil with phagocytic vacuoles. Note their larger size. Other evidence of toxicity in this cell is the pyknotic nucleus.

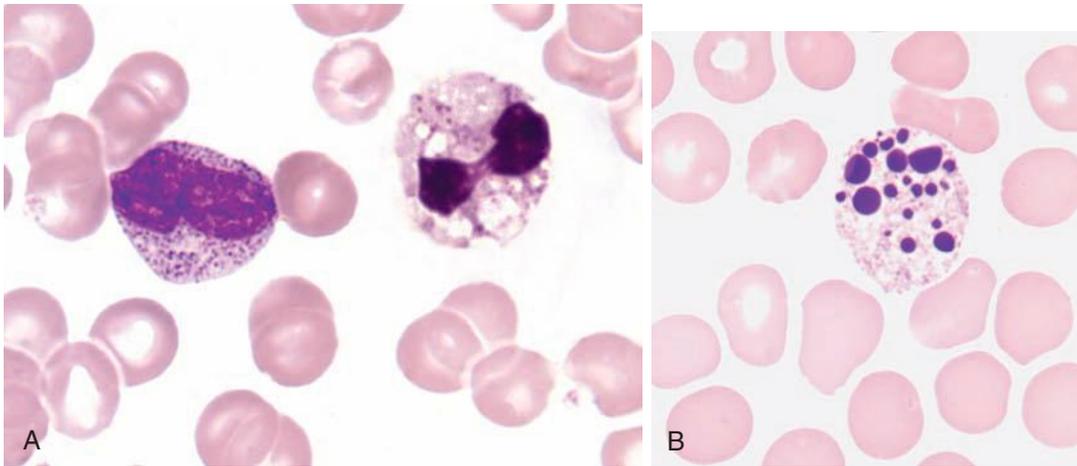


Figure 29-15 **A**, Upper cell is a neutrophil whose nucleus is dehydrated, which makes it very dark and dense. Note that there is still a filament between the segments. This is referred to as a pyknotic cell. The cell is also highly vacuolated. **B**, Neutrophil that has died. Note that the nucleus has disintegrated into numerous rounded spheres of DNA with no filaments. This is referred to as a necrotic or necrobiotic cell. (**B** from Carr JH, Rodak BF: *Clinical hematology atlas*, ed 3, St. Louis, 2009, Saunders.)

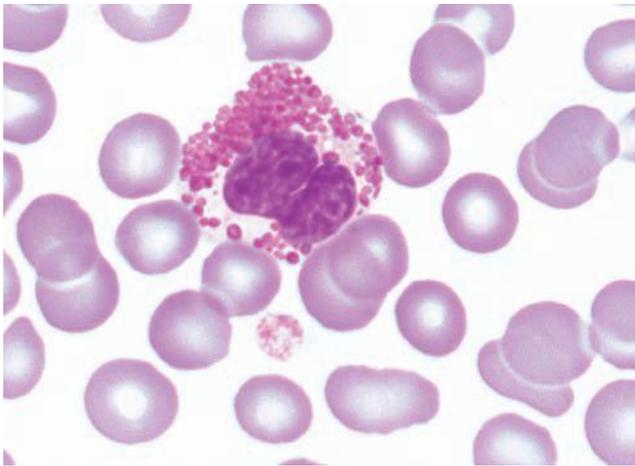


Figure 29-16 Partially degranulated eosinophil. This cell was found on the blood film for a patient with trichinosis.

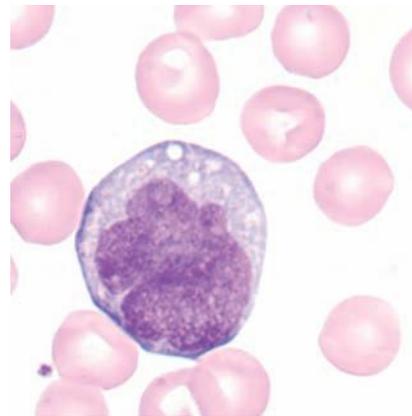


Figure 29-18 Immature monocyte. (From Carr JH, Rodak BF: *Clinical hematology atlas*, ed 4, St. Louis, 2013, Saunders.)

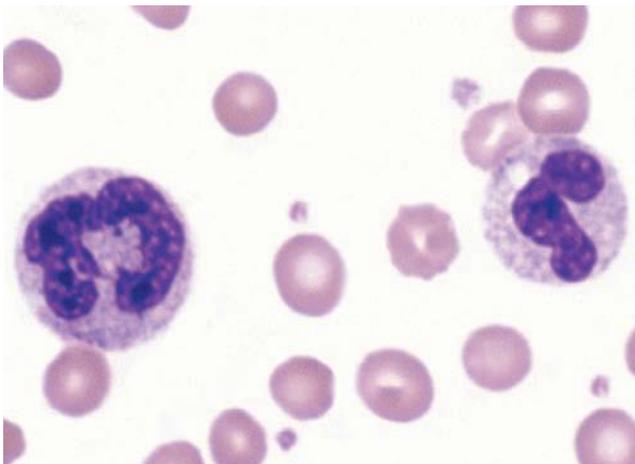


Figure 29-17 Neutrophil anisocytosis. The neutrophil to the left is larger than the other neutrophil. This is often caused by cytoplasmic swelling.

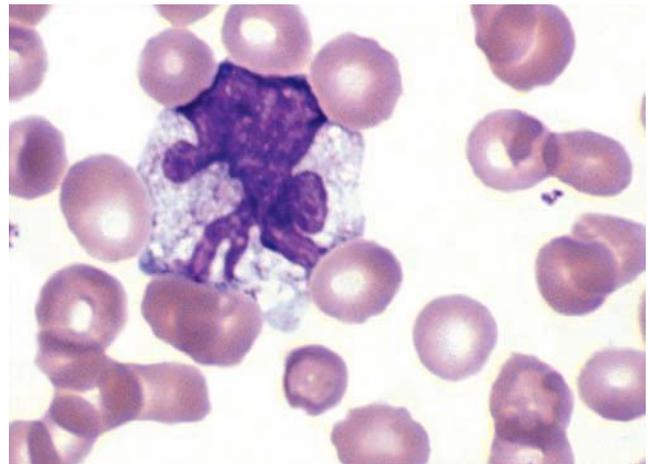


Figure 29-19 Reactive monocyte with contorted nucleus. Other evidence of toxicity is the several vacuoles in the cytoplasm.

(cytoplasmic vacuolation, intracellular debris, and irregular cytoplasmic borders) (Box 29-5B). Large numbers of immature monocytes occur most often in hematologic neoplasms involving the monocytic series.

Lymphocytes

Over the years, reactive morphologic changes in lymphocytes have been described using various terms, including *variant lymphocytes*, *reactive lymphocytes*, *effector lymphocytes*, *transformed lymphocytes*, *Turk cells*, *Downey cells*, *immunoblasts*, and *atypical lymphocytes*. *Atypical* is commonly used, but it is probably the least suitable of all because it implies that the cells are abnormal when in fact the lymphocytes are reacting to antigen in a normal manner. Also, the term *atypical*, when used by the cytology lab in a Pap smear result, suggests a suspicious, possibly precancerous lesion. Regardless of the labels that are applied, it is very important that the clinical staff understand the meaning behind the terms the lab uses to describe the reactive and malignant-appearing lymphocytes reported in the WBC differential.

Reactive lymphocytes are the result of complex morphologic and biochemical events that occur as lymphocytes are stimulated when interacting with antigens in peripheral lymphoid organs (Figures 29-20 and 29-21). B and T lymphocyte activation results in the transformation of small, resting lymphocytes into proliferating larger cells. These lymphocytes spill into peripheral circulation, which is what is encountered upon smear review. Reactive lymphocytes often present as a heterogeneous population of various shapes and sizes. There is variation in the nuclear/cytoplasmic ratio, nuclear shape, and the chromatin pattern, which is generally clumped, but some cells may demonstrate chromatin patterns that are less mature (less clumped). Nucleoli may be visible. Most obvious in reactive lymphocytes is an increase in basophilic cytoplasm that may vary in intensity within and between cells. The cytoplasm may be indented by surrounding RBCs, but it is important to realize that other cells, including blasts, may also show similar indentation.

Box 29-5B Reactive Morphologic Changes in Monocytes

Morphology	Associated with
Thin and band-like, or segmentation of nucleus; increased cytoplasmic volume and granulation, and/or evidence of phagocytic activity (cytoplasmic vacuolation, intracellular debris, and irregular cytoplasmic borders)	Infection, recovery from bone marrow aplasia, and granulocyte monocyte colony stimulating factor (GM-CSF) administration

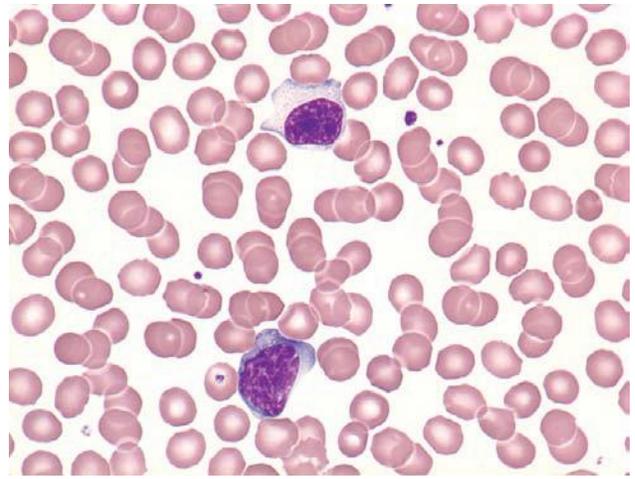


Figure 29-21 Reactive (variant) lymphocytes from a patient with infectious mononucleosis.

A *plasmacytoid lymphocyte* is a type of reactive lymphocyte that has some of the morphologic features of plasmacytes (Figure 29-22). However, because reactive lymphocytes may be activated T or B cells, it is important to understand that *plasmacytoid* is a morphologic term and does not imply lineage. Features of reactive lymphocytes are summarized in Box 29-5C.

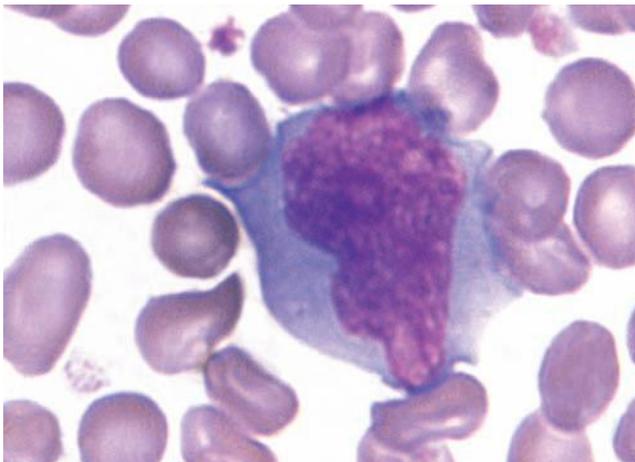


Figure 29-20 Reactive (variant) lymphocyte.

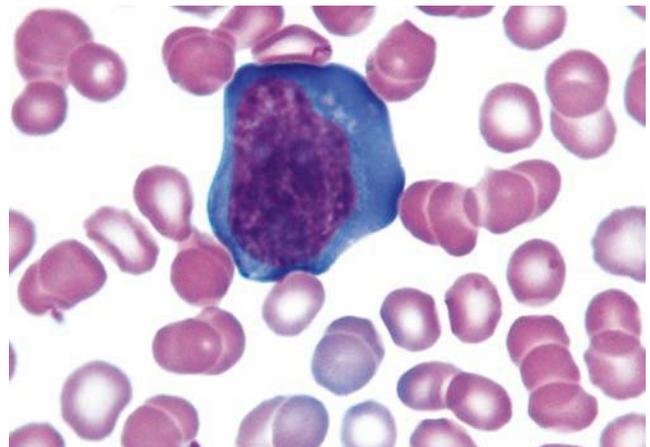


Figure 29-22 Reactive (variant) lymphocyte (plasmacytoid).

Box 29-5C Morphologic Changes in Reactive Lymphocytes

- Heterogeneous population of various shapes and sizes.
- Cells exhibit increased amount of variably basophilic cytoplasm.
- Lymphocyte population exhibits variation in nuclear/cytoplasmic ratio and/or nuclear shape.
- Chromatin is usually clumped however some cells may demonstrate less mature (less clumped) pattern.
- Nucleoli may be visible.
- The cytoplasm may be indented by surrounding RBCs

Epstein-Barr Virus (EBV)-Related Infections

Most humans are subclinically infected with EBV, which has been associated with several benign and malignant diseases but has only been proven to be the causative agent in a few, including infectious mononucleosis.

INFECTIOUS MONONUCLEOSIS (IM)

When primary infection with Epstein-Barr virus occurs in childhood, it often goes unnoticed. Infectious mononucleosis is the symptomatic illness that ensues whenever adolescents and adults are infected. The incubation period of infectious mononucleosis is about 3 to 7 weeks, and during this time the virus preferentially infects B lymphocytes through attachment of viral envelope glycoprotein 350/220 to CD21 (C3d complement receptors).¹¹⁴ The oropharynx epithelial cells are also infected, but the mechanism is unclear because these cells do not express CD21. The cellular response in IM is important in the control of the infection and is characterized by proliferation and activation of natural killer (NK) lymphocytes, CD4⁺ T cells, and CD8⁺ memory cytotoxic T cells (EBV-CTLs) in response to B cell infection. Most of the circulating reactive lymphocytes seen in circulation represent activated T cells.

Common clinical manifestations include sore throat, dysphagia, fever, chills, cervical lymphadenopathy, fatigue, and headache. Hematologic findings resemble those seen in many viral disorders. The WBC count is usually elevated to a range of $10\text{--}30 \times 10^9/\text{L}$ or more with an absolute lymphocytosis. There is a wide variation in lymphocyte morphology, with up to 50% or higher exhibiting reactive features. Complications that may

Box 29-6 Infectious Mononucleosis: Clinical and Lab Findings

Clinical Manifestations	Laboratory Test Results
<ul style="list-style-type: none"> • Common <ul style="list-style-type: none"> • Sore throat • Dysphagia • Fever • Chills • Cervical lymphadenopathy • Fatigue • Headache • Less common <ul style="list-style-type: none"> • Hepatomegaly • Elevated transaminases • Splenomegaly • Hemolytic anemia • Thrombocytopenia 	<ul style="list-style-type: none"> • WBC: $10\text{--}30 \times 10^9/\text{L}$ due to an absolute lymphocytosis • Reactive lymphocyte morphology • Positive heterophile antibody test • Positive EBV specific antigen & antibody tests

occur are generally mild and include hepatosplenomegaly (and elevated transaminases), hemolytic anemia, and moderate thrombocytopenia. In rare cases patients may develop aplastic anemia, disseminated intravascular coagulation, thrombotic thrombocytopenic purpura, hemolytic uremic syndrome, Guillain-Barré syndrome, or other neurologic complications.¹¹⁵ The incidence of IM in the United States is 500 cases per 100,000 annually. It has its highest frequency in young adults, aged 15 to 24 years,¹¹⁶ although infections have been reported in patients 3 months to 70 years of age.

Testing for infectious mononucleosis includes rapid screening tests for the detection of heterophile antibodies, antibodies stimulated by the EBV that cross-react with antigens found on sheep and horse red cells. However, not everyone with infectious mononucleosis will produce heterophile antibodies, especially children. Definitive testing for EBV infection includes a panel of antigen and antibody tests for VCA, EBNA, and IgG/IgM antibodies against VCA and EBNA. Cytomegalovirus is capable of causing a mononucleosis syndrome with similar clinical features.

Clinical and laboratory findings associated with infectious mononucleosis are summarized in [Box 29-6](#).

SUMMARY

- Pelger-Huët anomaly is a genetic disorder resulting in hypolobulated mature leukocytes. These cells can be confused with immature neutrophils. An acquired form of hyposegmentation called *pseudo-PHA* can be seen in some malignant myeloproliferative neoplasms.
- Alder-Reilly anomaly is a manifestation of the mucopolysaccharidosis characterized by metachromatic granules in leukocytes, which can be confused with toxic granulation.
- Chediak-Higashi syndrome is an inherited lethal disorder characterized by giant lysosomes in granular cells and dysfunctional leukocytes.
- May-Hegglin anomaly is characterized by thrombocytopenia, giant platelets, and Döhle body–like inclusions in leukocytes. Most affected individuals are asymptomatic.
- Chronic granulomatous disease is an inherited disorder of the NADPH oxidase system resulting in neutrophils that are incapable

of killing many microorganisms due to a failure in the respiratory burst, which is necessary to produce antibacterial agents.

- Leukocyte adhesion disorders are a group of disorders caused by mutations in the genes for adhesive molecules required for cells to migrate from the blood into the tissues.
- The mucopolysaccharidoses are a group of disorders, each of which is associated with a specific defect in an enzyme necessary for the degradation of GAGs such as heparan sulfate, keratan sulfate, dermatan sulfate, and chondroitin sulfate. The result is the buildup of partially digested GAGs within macrophages and leukocytes and clinical symptoms.
- The lipid storage diseases are a group of disorders, each of which is associated with a specific defect in an enzyme necessary for the degradation of lipids. The two lipid storage diseases with characteristic macrophage morphology are Gaucher disease and Niemann-Pick disease.

- Inherited lymphocyte disorders include DiGeorge syndrome, in which the lack or underdevelopment of the thymus results in decreased T cells; sex-linked agammaglobulinemia, in which the lack of a kinase results in blocked B cell development; and two types of severe combined immunodeficiency. Wiskott-Aldrich syndrome is a third inherited disorder affecting both T and B cells.
- Reactive changes in granulocytes include a left shift, Döhle bodies, toxic granulation, vacuoles, degranulation, and cytoplasmic swelling.
- Reactive changes in monocytes include occasional immature forms, contorted nuclei, and the presence of more immature forms.
- Reactive changes in lymphocytes include cell enlargement, increased basophilic cytoplasm, and morphologic heterogeneity.

Now that you have completed this chapter, go back and read again the case studies at the beginning and respond to the questions presented.

REVIEW QUESTIONS

Answers can be found in the Appendix.

- Which of the following inherited leukocyte disorders is caused by a mutation in the lamin B receptor?
 - Pelger-Huët anomaly
 - Chédiak-Higashi disease
 - Alder-Reilly anomaly
 - May-Hegglin anomaly
- Which of the following inherited leukocyte disorders is one of a group of disorders with mutations in nonmuscle myosin heavy-chain IIA?
 - Pelger-Huët anomaly
 - Chédiak-Higashi disease
 - Alder-Reilly anomaly
 - May-Hegglin anomaly
- Which of the following inherited leukocyte disorders might be seen in Hurler syndrome?
 - Pelger-Huët anomaly
 - Chédiak-Higashi disease
 - Alder-Reilly anomaly
 - May-Hegglin anomaly
- Which of the following lysosomal storage diseases is characterized by macrophages with striated cytoplasm and storage of glucocerebroside?
 - Sanfilippo syndrome
 - Gaucher disease
 - Fabry disease
 - Niemann-Pick disease
- The neutrophils in chronic granulomatous disease are incapable of producing:
 - Hydrogen peroxide
 - Hypochlorite
 - Superoxide
 - All of the above
- Individuals with X-linked SCID have a mutation that affects their ability to synthesize:
 - Deaminase
 - Oxidase
 - IL-2 receptor
 - IL-8 receptor
- An absolute lymphocytosis with reactive lymphocytes suggests which of the following conditions?
 - DiGeorge syndrome
 - Bacterial infection
 - Parasitic infection
 - Viral infection
- What leukocyte cytoplasmic inclusion is composed of ribosomal RNA?
 - Primary granules
 - Toxic granules
 - Döhle bodies
 - Howell-Jolly bodies

9. The expected complete blood count (CBC) results for women in active labor would include:
 - a. High total white blood cell (WBC) count with increased lymphocytes
 - b. High total WBC count with a slight shift to the left in neutrophils
 - c. Normal WBC count with increased eosinophils
 - d. Low WBC count with increased monocytes
10. Which of the following is true of an absolute increase in lymphocytes with reactive morphology?
 - a. The population of lymphocytes appears morphologically homogeneous.
 - b. They are usually effector B cells.
 - c. The reactive lymphocytes have increased cytoplasm with variable basophilia.
 - d. They are most commonly seen in bacterial infections.

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30

Cytogenetics

Gail H. Vance

OUTLINE

Reasons for Chromosome Analysis

Chromosome Structure

Cell Cycle

Chromosome Architecture

Metaphase Chromosomes

Chromosome Identification

Chromosome Number

Chromosome Size and Type

Techniques for Chromosome Preparation and Analysis

Chromosome Preparation

Chromosome Banding

Metaphase Analysis

Fluorescence In Situ Hybridization

Cytogenetic Nomenclature

Chromosome Abnormalities

Numeric Abnormalities

Structural Abnormalities

Cancer Cytogenetics

Leukemia

Solid Tumors

Chromosomal Microarray Analysis

OBJECTIVES

After completion of this chapter, the reader will be able to:

1. Describe chromosome structure and the methods used in G-banded chromosome identification.
2. Explain the basic laboratory techniques for preparing chromosomes for analysis.
3. Differentiate between numeric and structural chromosome abnormalities.
4. Discuss the importance of karyotype in the diagnosis of hematologic cancer.
5. Explain the basic technique of fluorescence in situ hybridization (FISH).
6. Discuss the advantage of using FISH analysis in conjunction with G-banded analysis of cells.
7. Describe the types of chromosomal abnormalities that are detectable with cytogenetic methods.
8. Given a diagram of a G-banded chromosome, name the structures identifiable by light microscopy.
9. Given the designation of a chromosome mutation, be able to determine whether the abnormality is numeric or structural, which chromosomes are affected, what type of abnormality it is, and what portion of the chromosome is affected.
10. Define chromosomal microarray analysis.

CASE STUDY

After studying the material in this chapter, the reader should be able to respond to the following case study:

A 54-year-old man came to his physician with a history of fatigue, weight loss, and increased bruising over a 6-month period. His WBC count was elevated at $200 \times 10^9/L$. A bone marrow aspirate was sent for cytogenetic analysis. G-banded chromosome analysis of 20 cells from bone marrow cultures showed all cells to be positive for the Philadelphia chromosome, $t(9;22)(q34;q11.2)$, as seen in chronic myelogenous leukemia (Figure 30-1). FISH studies using



Figure 30-1 Karyogram for the patient in the case study showing a translocation between chromosomes 9 and 22, which is characteristic of chronic myelogenous leukemia. (Courtesy the Cytogenetics Laboratory, Indiana University School of Medicine, Indianapolis, IN.)

CASE STUDY—cont'd

After studying the material in this chapter, the reader should be able to respond to the following case study:

the *BCR* and *ABL1* gene probes (Abbott Molecular, Des Plaines, IL) produced dual fusion signals, one located on the derivative chromosome 9 and one on the derivative chromosome 22, characteristic of the translocation between chromosomes 9 and 22 leading to the rearrangement of *BCR* and *ABL1* oncogenes (Figure 30-2). The patient was treated with imatinib mesylate for the next 2 months. Another cytogenetic study was performed on a second bone marrow aspirate. This analysis showed that 12 of 20 cells analyzed were normal, 46,XY[12]; however, there were still 8 cells positive for the Philadelphia chromosome, 46,XY,t(9;22)(q34; q11.2)[8].

1. What is G-banded chromosome analysis?
2. Is the described mutation an example of a numeric or a structural abnormality? What type? Which chromosomes are involved? Explain.
3. What is FISH, and how does it complement standard chromosome analysis?

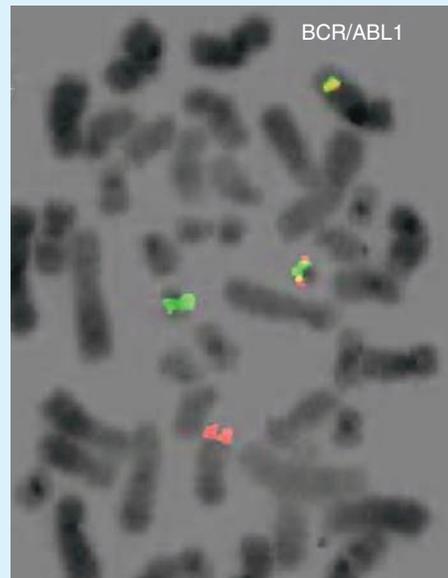


Figure 30-2 Bone marrow metaphase cell from the patient in the case study hybridized with probes for *BCR* (green) and *ABL1* (red) (Abbott Molecular, Des Plaines, IL). The fusion signals (yellow) represent the translocated chromosomes 9 and 22 (der(9) and der(22)). (Courtesy the Cytogenetics Laboratory, Indiana University School of Medicine, Indianapolis, IN.)

Human cytogenetics is the study of chromosomes, their structure, and their inheritance. There are approximately 25,000 genes in the human genome, most of which reside on the 46 chromosomes normally found in each somatic cell.¹

Chromosome disorders are classified as structural or numerical and involve the loss, gain, or rearrangement of either a piece of a chromosome or the entire chromosome. Because each chromosome contains thousands of genes, a chromosomal abnormality that is observable by light microscopy involves, on average, 3 to 5 megabases (Mb) of DNA and represents the disruption or loss of hundreds of genes. Such disruptions often have a profound clinical effect. Chromosomal abnormalities are observed in approximately 0.65% of all live births.² The gain or loss of an entire chromosome, other than a sex chromosome, is usually incompatible with life and accounts for approximately 50% of first-trimester spontaneous abortions.³ In leukemia, cytogenetic abnormalities are observed in more than 50% of bone marrow specimens.⁴ These recurring abnormalities often define the leukemia and frequently indicate clinical prognosis.

REASONS FOR CHROMOSOME ANALYSIS

Chromosome analysis is an important diagnostic procedure in clinical medicine. Not only are chromosomal anomalies major causes of reproductive loss and birth defects, but also nonrandom

chromosome abnormalities are recognized in many forms of cancer.

Physicians who care for patients of all ages may order chromosome analysis or karyotyping for patients with mental retardation, infertility, ambiguous genitalia, short stature, fetal loss, risk of genetic or chromosomal disease, and cancer (Table 30-1). In the following discussion, basic cytogenetic concepts are presented. Supplementation of this chapter with the material in Chapter 31 is recommended.

CHROMOSOME STRUCTURE

Cell Cycle

The cell cycle is divided into four stages: G_1 , the growth period before synthesis of deoxyribonucleic acid (DNA); S phase, the period during which DNA synthesis takes place; G_2 , the period after DNA synthesis; and M, the period of mitosis or cell division, the shortest phase of the cell cycle (Figure 6-5). During mitosis, chromosomes are maximally condensed. While in mitosis, cells can be chemically treated to arrest cell progression through the cycle so that the chromosomes may be isolated and analyzed.

Chromosome Architecture

A chromosome is formed from a double-stranded DNA molecule that contains a series of genes. The complementary

TABLE 30-1 Common Translocations in Hematopoietic and Lymphoid Neoplasia and Sarcoma*

Tumor Type	Karyotype	Genes
Myeloid Leukemias		
CML (and pre-B-ALL)	t(9;22)(q34;q11.2)	<i>BCR/ABL1</i>
Also see Box 31-1		
B Cell Leukemias/Lymphomas		
B lymphoblastic leukemia	t(12;21)(p13;q22)	<i>ETV6/RUNX1</i>
	t(1;19)(q23.3;p13.3)	<i>PBX1/TCF3</i>
	t(4;11)(q21;q23)	<i>AFF1/MLL(KMT2A)</i>
Burkitt lymphoma	t(8;14)(q24;q32.3)	<i>MYC/IGH</i>
	t(2;8)(p12;q24)	<i>IGK/MYC</i>
	t(8;22)(q24;q11.2)	<i>MYC/IGL</i>
Mantle cell lymphoma	t(11;14)(q13;q32.3)	<i>CCND1/IGH</i>
Follicular lymphoma	t(14;18)(q32.3;q21.3)	<i>IGH/BCL2</i>
Diffuse large B cell lymphoma	t(3;14)(q27;q32.3)	<i>BCL6/IGH</i>
Lymphoplasmacytic lymphoma	t(9;14)(p13.2;q32.3)	<i>PAX5/IGH</i>
MALT lymphoma	t(14;18)(q32.3;q21)	<i>IGH/MALT1</i>
	t(11;18)(q22;q21)	<i>BIRC3/MALT1</i>
	t(1;14)(p22;q32.3)	<i>BCL10/IGH</i>
T Cell Leukemias/Lymphomas		
T lymphoblastic leukemia	del(1)(p32p32)	<i>STIL/TAL1</i>
	t(7;11)(q34;p13)	<i>TRB/LMO2</i>
ALCL	t(2;5)(p23;q35.1)	<i>ALK/NPM1</i>
Sarcomas and Tumors of Bone and Soft Tissue		
Alveolar rhabdomyosarcoma	t(2;13)(q36.1;q14.1)	<i>PAX3/FOXO1A</i>
	t(1;13)(p36.13;q14.1)	<i>PAX7/FOXO1A</i>
Ewing sarcoma/PNET	t(11;22)(q24;q12.2)	<i>FLI1/EWSR1</i>
	t(21;22)(q22.3;q12.2)	<i>ERG/EWSR1</i>
	t(7;22)(p22;q12.2)	<i>ETV1/EWSR1</i>
Clear cell sarcoma	t(12;22)(q13;q12.2)	<i>ATF1/EWSR1</i>
Myxoid liposarcoma	t(12;16)(q13;p11.2)	<i>DDIT3/FUS</i>
	t(12;22)(q13;q12.2)	<i>DDIT3/EWSR1</i>
Synovial sarcoma	t(X;18)(p11.2;q11.2)	<i>SSX1 or SSX2/SS18</i>
Alveolar soft part sarcoma	t(X;17)(p11.2;q25)	<i>TFE3/ASPCR1</i>

ALCL, Anaplastic large cell leukemia; *ALL*, acute lymphoblastic leukemia; *AML*, acute myeloid leukemia; *CML*, chronic myelogenous leukemia; *CMML*, chronic myelomonocytic leukemia; *MALT*, mucosa-associated lymphoid tissue; *PNET*, primitive neuroectodermal tumor.

*Modified per the Hugo Nomenclature Database, May 2013

double-helix structure of DNA was established in 1953 by Watson and Crick.⁵ The backbone is a sugar-phosphate-sugar polymer. The sugar is deoxyribose. Attached to the backbone and filling the center of the helix are four nitrogen-containing bases. Two of these, adenine (A) and guanine (G), are purines; the other two, cytosine (C) and thymine (T), are pyrimidines (Figure 31-6).

The chromosomal DNA of the cell resides in the cell's nucleus. This DNA and its associated proteins are referred to as *chromatin*. During the cell cycle, at mitosis, the nuclear chromatin condenses approximately 10,000-fold to form

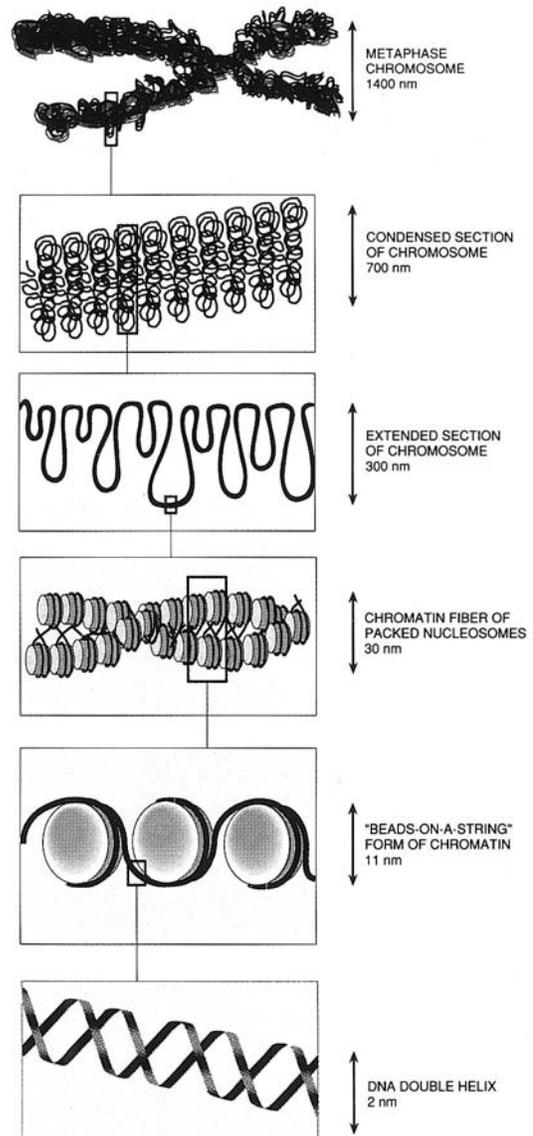


Figure 30-3 Chromosome structure. The folding and twisting of the DNA double helix. (From Gelehrter TD, Collins FS, Ginsberg D: *Principles of medical genetics*, ed 2, Philadelphia, 1998, Lippincott Williams & Wilkins.)

chromosomes.⁶ Each chromosome results from progressive folding, compression, and compaction of the entire nuclear chromatin. This condensation is achieved through multiple levels of helical coiling and supercoiling (Figure 30-3).

Metaphase Chromosomes

Metaphase is the stage of mitosis where the chromosomes align on the equatorial plate. Electron micrographs of metaphase chromosomes have provided models of chromosome structure. In the “beads-on-a-string” model of chromatin folding, the DNA helix is looped around a core of histone proteins.⁷ This packaging unit is known as a *nucleosome* and measures approximately 11 nm in diameter.⁸ Nucleosomes are coiled into twisted forms to create an approximately 30-nm

chromatin fiber. This fiber, called a *solenoid*, is condensed further and bent into a loop configuration. These loops extend at an angle from the main chromosome axis.⁹

CHROMOSOME IDENTIFICATION

Chromosome Number

In 1956, Tijo and Levan¹⁰ identified the correct number of human chromosomes as 46. This is the *diploid* chromosome number and is determined by counting the chromosomes in dividing somatic cells. The designation for the diploid number is $2n$. Gametes (ova and sperm) have half the diploid number (23). This is called the *haploid* number of chromosomes and is designated as n . Different species have different numbers of chromosomes. The reindeer has a relatively high chromosome number for a mammal ($2n = 76$), whereas the Indian muntjac, or barking deer, has a very low chromosome number ($2n = 7$ in the male and $2n = 6$ in the female).¹¹

Chromosome Size and Type

In the 1960s, before the discovery of banding, chromosomes were categorized by overall size and the location of the centromere (primary constriction) and were assigned to one of seven groups: A through G. Group A includes chromosome pairs 1, 2, and 3. These are the largest chromosomes, and their centromeres are located in the middle of the chromosome; that is, they are metacentric. Group B chromosomes, pairs 4 and 5, are the next largest chromosomes; their centromeres are off center, or submetacentric. Group G consists of the smallest chromosomes, pairs 21 and 22, whose centromeres are located at one end of the chromosomes and are designated as acrocentric (Figure 30-4).

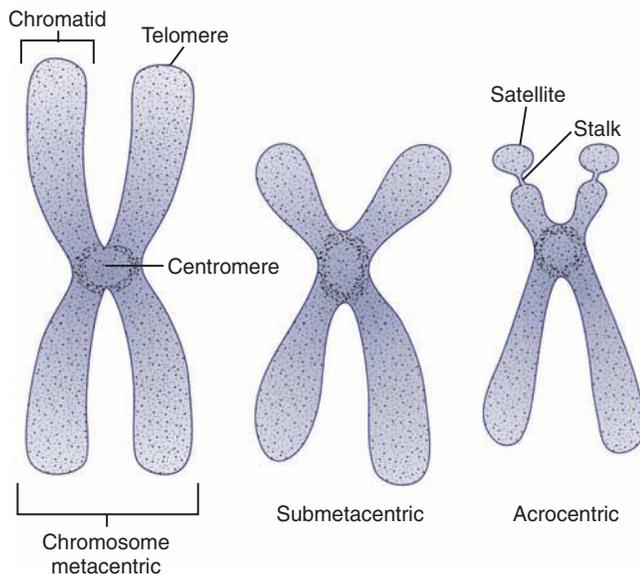


Figure 30-4 Chromosome morphology. The three shapes of chromosomes are metacentric, submetacentric, and acrocentric. This figure also shows the position of the centromere and telomere as well as the two sister chromatids that comprise a metaphase chromosome.

TECHNIQUES FOR CHROMOSOME PREPARATION AND ANALYSIS

Chromosome Preparation

Tissues used for chromosome analysis contain cells with an inherently high mitotic rate (bone marrow cells) or cells that can be stimulated to divide in culture (peripheral blood lymphocytes). Special harvesting procedures are established for each tissue type. Mitogens such as phytohemagglutinin or pokeweed mitogen are added to peripheral blood cultures. Phytohemagglutinin primarily stimulates T cells to divide,¹² whereas pokeweed preferentially stimulates B lymphocytes.¹³

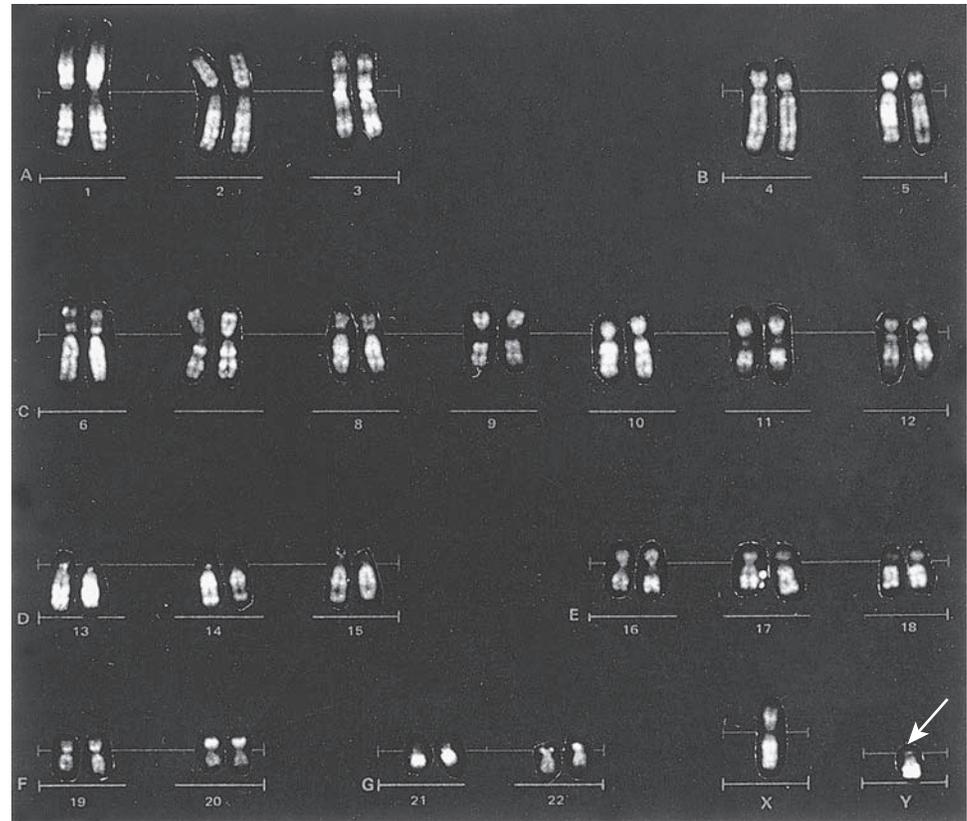
Chromosomes may be obtained from replicating cells by arresting the cell in metaphase. Cells from the peripheral blood or bone marrow are cultured in media for 24 to 72 hours. In standard peripheral blood cultures, since the cells are terminally differentiated, a mitogen is added to stimulate cellular division. Neoplastic cells are spontaneously dividing and generally do not require stimulation with a mitogen. After the cell cultures have grown for the appropriate period, Colcemid, an analogue of colchicine, is added to disrupt the mitotic spindle fiber attachment to the chromosome. Following culture and treatment with Colcemid, cells are exposed to a hypotonic (potassium chloride) solution that lyses red cells and causes the chromosomes to spread apart from one another. A fixative of 3:1 methanol and acetic acid is added that “hardens” cells and removes proteinaceous material. Cells are dropped onto cold, wet glass slides to achieve optimal dispersal of the chromosomes. The slides are then aged, typically by exposure to heat, before banding.

Chromosome Banding

Analysis of each chromosome is made possible by staining with a dye. The name *chromosome* is derived from the Greek words *chroma*, meaning “color,” and *soma*, meaning “body.” Hence *chromosome* means “colored body.” In 1969, Caspersen and colleagues¹⁴ were the first investigators to stain chromosomes successfully with a fluorochrome dye. Using quinacrine mustard, which binds to adenine-thymine-rich areas of the chromosome, they were able to distinguish a banding pattern unique to each chromosome. This banding pattern, called *Q-banding*, differentiates the chromosome into bands of differing widths and relative brightnesses (Figure 30-5). The most brightly fluorescent bands of the 46 human chromosomes include the distal end of the Y chromosome, the centromeric regions of chromosomes 3 and 4, and the short arms of the acrocentric chromosomes (13, 14, 15, 21, and 22).

Other stains are used to identify chromosomes, but in contrast to Q-banding, these methods normally necessitate some pretreatment of the slide to be analyzed. Giemsa (G) bands are obtained by pretreating the chromosomes with the proteolytic enzyme trypsin. *GTG banding* means “G banding by Giemsa with the use of trypsin.” Giemsa, like quinacrine mustard, stains AT-rich areas of the chromosome. The dark bands are called *G-positive* (+) bands. Guanine-cytosine-rich areas of the chromosome have little affinity for the dye and are referred to

Figure 30-5 Q-banded preparation. Note the intense brilliance of Yq. (Courtesy the Cytogenetics Laboratory, Indiana University School of Medicine, Indianapolis, IN.)



as *G-negative* (–) bands. G+ bands correspond with the brightly fluorescing bands of Q-banding (Figures 30-6 and 30-7). G-banding is the most common method used for staining chromosomes.

C-banding stains the centromere (primary constriction) of the chromosome and the surrounding condensed heterochromatin. Constitutive heterochromatin is a special type of late-replicating repetitive DNA that is located primarily at the centromere of the chromosome. In C-banding, the chromosomes are treated first with an acid and then with an alkali (barium hydroxide) before Giemsa staining. C-banding is most intense in human chromosomes 1, 9, and 16 and the Y chromosome.

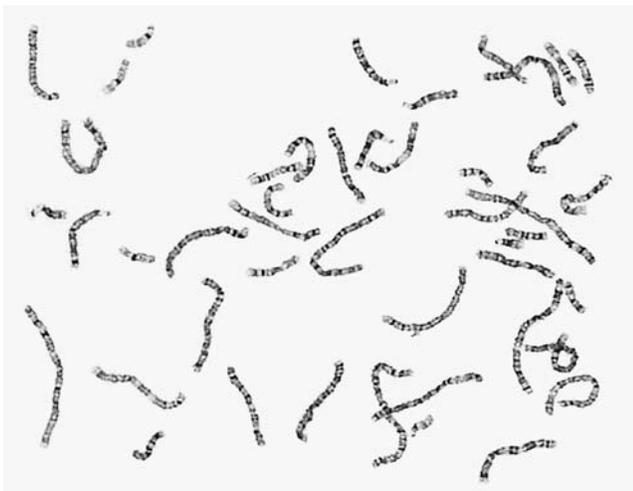


Figure 30-6 Normal male metaphase chromosomes.



Figure 30-7 Normal male karyogram, GTG-banded preparations. (Courtesy the Cytogenetics Laboratory, Indiana University School of Medicine, Indianapolis, IN.)

Polymorphisms from different individuals are also observed in the C-bands. These polymorphisms have no clinical significance (Figure 30-8).

Specific chromosomal regions that are associated with the nucleoli in interphase cells are called *nucleolar organizer regions* (NORs). NORs contain tandemly repeated ribosomal nucleic acid (RNA) genes. NORs can be differentially stained in chromosomes by a silver stain in a method called *AG-NOR-banding*.

Chromosome banding is visible after chromosome condensation, which occurs during mitosis. The banding pattern

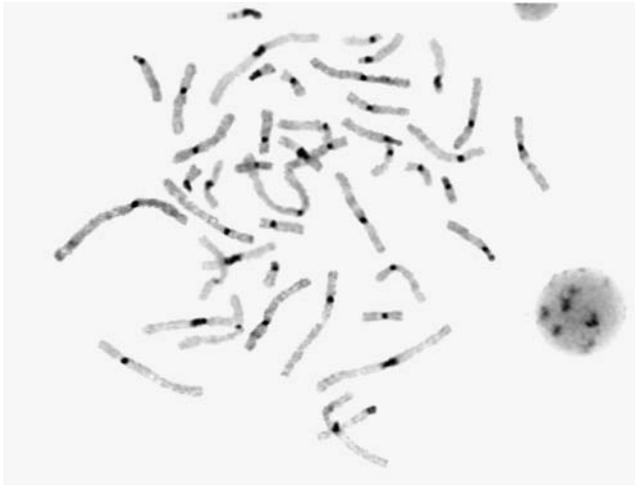


Figure 30-8 C-banded male metaphase chromosomes. Note the stain at the centromere and heterochromatic regions of the chromosomes. (Courtesy the Cytogenetics Laboratory, Indiana University School of Medicine, Indianapolis, IN.)

observed depends on the degree of condensation. By examination of human chromosomes early in mitosis, it has been possible to estimate a total haploid genome (23 chromosomes) with approximately 2000 AT-rich (G+) bands.¹⁵ The later the stage of mitosis, the more condensed the chromosome and the fewer total G+ bands observed.

Metaphase Analysis

After banding, prepared slides with dividing cells are scanned under a light microscope with a low-power objective lens (10 \times). When a metaphase cell has been selected for analysis, a 63 \times or 100 \times oil immersion objective lens is used. Each metaphase cell is analyzed first for a chromosome number. Then each chromosome pair is analyzed for its banding pattern. A normal somatic cell contains 46 chromosomes, which

includes two sex chromosomes and 22 pairs of autosomes (chromosomes 1 through 22). The technologist records his or her summary of the analysis using chromosome nomenclature. This summary is called a *karyotype*. Any variation in number and banding pattern is recorded by the technologist. At least 20 metaphase cells are analyzed from leukocyte cultures. If abnormalities are noted, the technologist may need to analyze additional cells. Computer imaging or photography is used to confirm and record the microscopic analysis. A picture of all the chromosomes aligned from 1 to 22 including the sex chromosomes is called a *karyogram*.

Fluorescence In Situ Hybridization

The use of molecular methods coupled with standard karyotype analysis has improved chromosomal mutation detection beyond that of the light microscope. DNA or RNA probes labeled with either fluorescent or enzymatic detection systems are hybridized directly to metaphase or interphase cells on a glass microscope slide. These probes usually belong to one of three classes: probes for repetitive DNA sequences, primarily generated from centromeric DNA; whole-chromosome probes that include segments of an entire chromosome; and specific loci or single-copy probes.

Fluorescence in situ hybridization (FISH) is a molecular technique commonly used in cytogenetic laboratories. FISH studies are a valuable adjunct to the diagnostic workup. In FISH, the DNA or RNA probe is labeled with a fluorophore. Target DNA is treated with heat and formamide to denature the double-stranded DNA, which renders it single-stranded. The target DNA anneals to a similarly denatured, single-stranded, fluorescently labeled DNA or RNA probe with a complementary sequence. After hybridization, the unbound probe is removed through a series of stringent washes, and the cells are counterstained for visualization (Figure 30-9).

In situ hybridization with centromere or whole-chromosome painting probes can be used to identify individual chromosomes

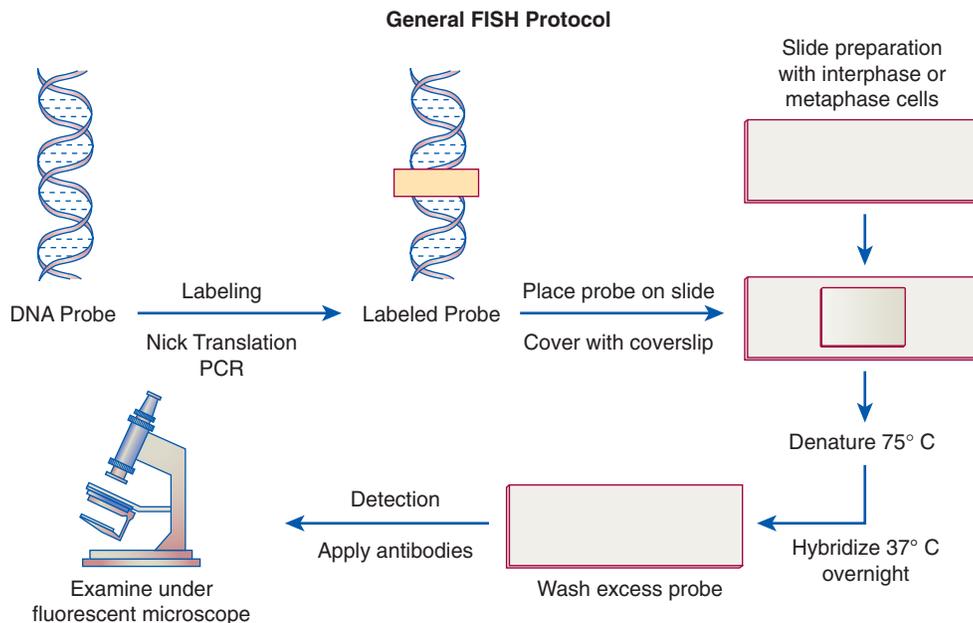


Figure 30-9 General protocol for fluorescence in situ hybridization. *PCR*, Polymerase chain reaction.

(Figure 30-10). Marker chromosomes represent chromatin material that has been structurally altered and cannot be identified by a G-band pattern. FISH using a centromere or paint probe, or both, is often helpful in identifying the chromosome of origin (Figure 30-11).¹⁶ Specific loci probes can be used to detect both structural and numerical abnormalities but are especially helpful in identifying chromosomal translocations or inversions.

The FISH procedure has many advantages and has advanced the detection of chromosomal abnormalities beyond that of G-banded analysis. Both dividing (metaphase) and nondividing (interphase) cells can be analyzed with FISH. Performance of FISH on uncultured cells, such as bone marrow smears, provides a quick test result that can be reported in 24 hours. Also, in cultured bone marrow samples submitted for G-band analysis, the number of dividing cells may be insufficient for cytogenetic diagnosis. In such cases, FISH performed on interphase (nondividing) cells with probes for a specific translocation or structural abnormality may provide the diagnosis. FISH also can be performed on paraffin-embedded tissue sections, specimens obtained by fine needle aspiration, and touch preparations from lymph nodes or solid tumors.

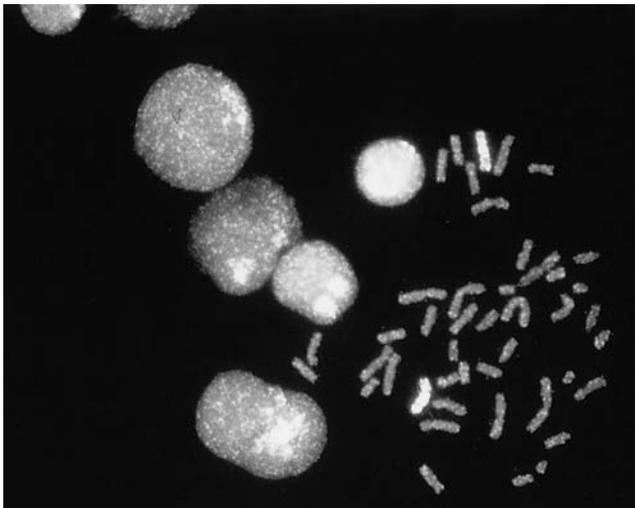


Figure 30-10 Metaphase preparation is “painted” with multiple probes for chromosome 7, producing a fluorescent signal. (Courtesy the Cytogenetics Laboratory, Indiana University School of Medicine, Indianapolis, IN.)

Locus-specific probes Chromosome paint probes Centromere probes	
Detection of numerical chromosome abnormalities	Detection of structural chromosome abnormalities
Trisomy Monosomy Polyploidy	Deletions Duplications Inversions Translocations Ring chromosomes Marker chromosomes Dicentric chromosomes

Figure 30-11 Fluorescence in situ hybridization in the clinical laboratory.

CYTOGENETIC NOMENCLATURE

Banding techniques enabled scientists to identify each chromosome pair by a characteristic banding pattern. In 1971, a Paris conference for nomenclature of human chromosomes was convened to designate a system to describe the regions and specific bands of the chromosomes. The chromosome arms were designated *p* (petite) for the short arm and *q* for the long arm. The regions in each arm and the bands contained within each region were numbered consecutively, from the centromere outward to the telomere or end of the chromosome. To designate a specific region of the chromosome, the chromosome number is written first, followed by the designation of either the short or long arm, then the region of the arm, and finally the specific band. *Xq21* designates the long arm of the X chromosome, region 2, band 1. To designate a subband, a decimal point is placed after the band designation, followed by the number assigned to the subband, as in *Xq21.1* (Figure 30-12).

Cytogenetic (and FISH) nomenclature represents a uniform code used by cytogeneticists around the world to communicate chromosome abnormalities. In this nomenclature each string begins with the modal number of chromosomes, followed by the sex chromosome designation. A normal male karyotype is designated 46,XY, and a normal female karyotype is designated 46,XX. If abnormalities are observed in the cell, the designation is written to include abnormalities of modal chromosome number, sex chromosomes, and then the autosomes. A cell from a bone marrow specimen with trisomy of chromosome 8 (three copies of chromosome 8) in a male is written as 47,XY,+8 (no intervening spaces). The number of cells with this abnormality is indicated in brackets. If 20 cells were examined, trisomy 8 was found in 10 cells, and the remainder were normal, the findings would be written as 47,XY,+8[10]/46,XY[10]. Translocations

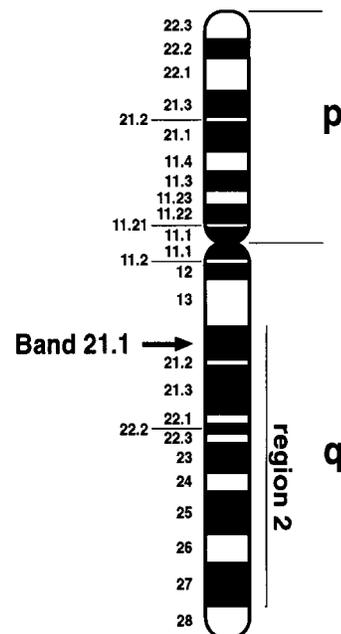


Figure 30-12 Banding pattern of the human X chromosome at the 550 average band level. Arrow indicates the location of Xq21.1.

(exchange of material between two chromosomes) are designated *t*, with the lowest chromosome number listed first. Thus a translocation between the short arm of chromosome 12 at band p13 and the long arm of chromosome 21 at band q22 is written as $t(12;21)(p13;q22)$. A semicolon is used to separate the chromosomes and the band designations. A translocated chromosome is called a *derivative chromosome*. Using the previous example, chromosomes 12 and 21 are referred to as *der(12)* and *der(21)*. Deletions are written with the abbreviation *del* preceding the chromosome. A deletion of the long arm of chromosome 5 at band 31 is written as $del(5)(q31)$. No spaces are entered in these designations except between abbreviations.¹⁷

CHROMOSOME ABNORMALITIES

There are many types of chromosome abnormalities, such as deletions, inversions, ring formations, trisomies, and polyploidy. All these defects can be grouped into two major categories: defects involving an abnormality in the *number* of chromosomes and defects involving *structural* changes in one or more chromosomes.

Numeric Abnormalities

Numeric abnormalities often are subclassified as aneuploidy or polyploidy. *Aneuploidy* refers to any abnormal number of chromosomes that is not a multiple of the haploid number (23 chromosomes). The common forms of aneuploidy in humans are trisomy (the presence of an extra chromosome) and monosomy (the absence of a single chromosome). Aneuploidy is the result of nondisjunction, the failure of chromosomes to separate normally during cell division. Nondisjunction can occur during either of the two types of cell division: mitosis or meiosis. During normal mitosis, a cell divides once to produce two cells that are identical to the parent cell. In mitosis, each daughter cell contains 46 chromosomes. Meiosis is a special type of cell division that generates male and female gametes (sperm and ova). In contrast to mitosis, meiosis entails two cell divisions: meiosis I and meiosis II. The end result is a cell with 23 chromosomes, which is the haploid number (*n*).

In polyploidy, the chromosome number is higher than 46 but is always an exact multiple of the haploid chromosome number of 23. A karyotype with 69 chromosomes is called *triploidy* ($3n$) (Figure 30-13). A karyotype with 92 chromosomes is called *tetraploidy* ($4n$).

In cancer, numerical abnormalities in the karyotype may be classified further based on the modal number of chromosomes in a neoplastic clone. *Hypodiploid* refers to a cell with fewer than 46 chromosomes; *near-haploid* cells have from 23 up to approximately 34 chromosomes (Figure 30-14); *hyperdiploid* cells have more than 46 chromosomes. *High hyperdiploidy* refers to a chromosome number of more than 50.¹⁸ Finally the term *pseudodiploid* is used to describe a cell with 46 chromosomes and structural abnormalities.

Structural Abnormalities

Structural rearrangements result from breakage of a chromosome region with loss or subsequent rejoining in an abnormal

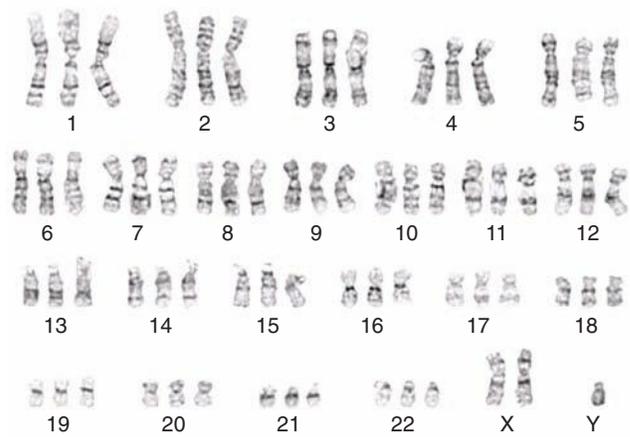


Figure 30-13 Triploid karyotype, 69,XXY. (Courtesy the Cytogenetics Laboratory, Indiana University School of Medicine, Indianapolis, IN.)



Figure 30-14 Hypodiploid karyotype with 36 chromosomes (arrows indicate missing chromosomes).

combination. Structural rearrangements are defined as *balanced* (no loss or gain of genetic chromatin) or *unbalanced* (gain or loss of genetic material). Structural rearrangements of single chromosomes include inversions, deletions, isochromosomes, ring formations, insertions, translocations, and duplications. Inversions (*inv*) involve one or two breaks in a single chromosome, followed by a 180-degree rotation of the segment between the breaks with no loss or gain of material. If the chromosomal material involves the centromere, the inversion is called *pericentric*. If the material that is inverted does not include the centromere, the inversion is called *paracentric* (Figure 30-15).

Interstitial *deletions* arise after two breaks in the same chromosome arm and loss of the segment between the breaks. Terminal deletions (loss of chromosomal material from the end of a chromosome) and interstitial deletions involve the loss of genetic material. The clinical consequence to the individual with a deletion depends on the extent and location of the deleted chromosomal material (Figure 30-16).

Isochromosomes arise from either abnormal division of the centromeres in which division is perpendicular to the long axis of the chromosome rather than parallel to it or from breakage and reunion in chromatin adjacent to the centromere. Each resulting daughter cell has a chromosome in which the short arm or the long arm is duplicated.

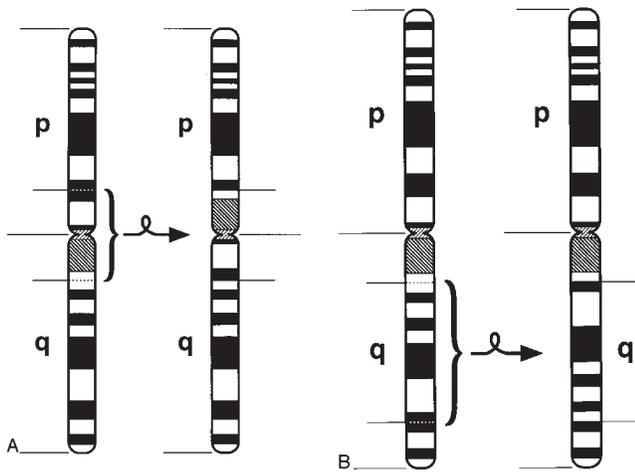


Figure 30-15 A, Pericentric inversion involves the centromere. B, Paracentric inversion occurs in either the short or long arm of the chromosome.

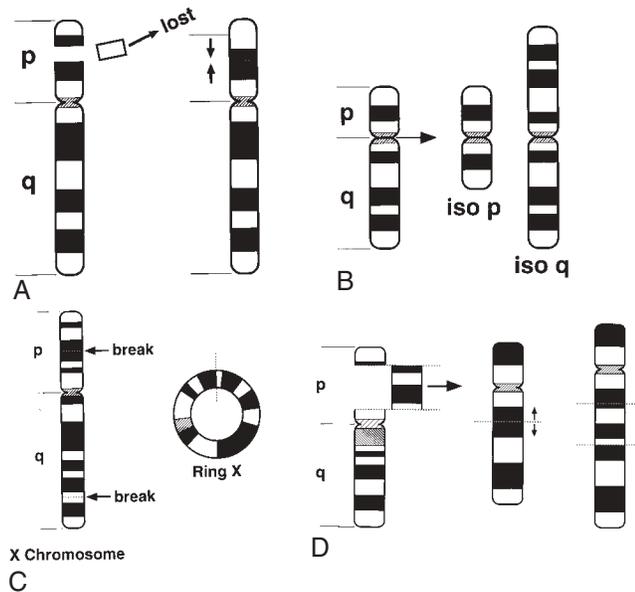


Figure 30-16 A, Interstitial deletion. B, Isochromosome. C, Ring chromosome. D, Insertion.

Ring chromosomes can result from breakage and reunion of a single chromosome with loss of chromosomal material outside the break points. Alternatively, one or both telomeres (chromosome ends) may join to form a ring chromosome without significant loss of chromosomal material.

Insertions involve movement of a segment of a chromosome from one location of the chromosome to another location of the same chromosome or to another chromosome. The segment is released as a result of two breaks, and the insertion occurs at the site of another break.

Duplication means partial trisomy for part of a chromosome. This can result from an unbalanced insertion or unequal crossing over in meiosis or mitosis.

Translocations occur when there is breakage in two chromosomes and each of the broken pieces reunites with another chromosome. If chromatin is neither lost nor gained, the

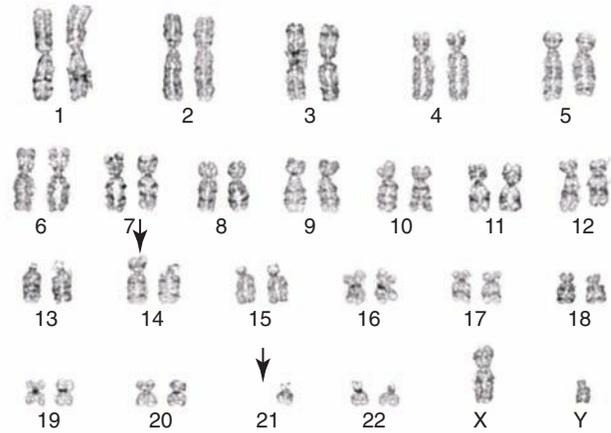


Figure 30-17 Balanced Robertsonian translocation between chromosomes 14 and 21. The nomenclature for this karyogram is written 45,XY,der(14;21)(q10;q10). (Courtesy the Cytogenetics Laboratory, Indiana University School of Medicine, Indianapolis, IN.)

exchange is called a *balanced reciprocal translocation*. A reciprocal translocation is balanced if all chromatin material is present. The loss or gain of chromatin material results in partial monosomy or trisomy for a segment of the chromosome, which is designated an *unbalanced rearrangement*.

Another type of translocation involving breakage and reunion near the centromeric regions of two acrocentric chromosomes is known as a *Robertsonian translocation*. Effectively this is a fusion between two whole chromosomes rather than exchange of material, as in a reciprocal translocation. These translocations are among the most common balanced structural rearrangements seen in the general population with a frequency of 0.09% to 0.1%.¹⁹ All five human acrocentric autosomes (13, 14, 15, 21, and 22) are capable of forming a Robertsonian translocation. In this case, the resulting balanced karyotype has only 45 chromosomes, which include the translocated chromosomes (Figure 30-17).

CANCER CYTOGENETICS

Cancer cytogenetics is a field that has been built upon discovery of nonrandom chromosome abnormalities in many types of cancer. In hematologic neoplasias, specific structural rearrangements are associated with distinct subtypes of leukemia that have characteristic morphologic and clinical features. Cytogenetic analysis of malignant cells can help determine the diagnosis and often the prognosis of a hematologic malignancy, assist the oncologist in the selection of appropriate therapy, and aid in monitoring the effects of therapy. Bone marrow is the tissue most frequently used to study the cytogenetics of a hematologic malignancy. Unstimulated peripheral blood and bone marrow trephine biopsy samples also may be analyzed. Cytogenetic analysis of cancers involving other organ systems can be performed using solid tissue obtained during surgery or by needle biopsy. Chromosomal defects in cancer include a wide range of numeric abnormalities and structural rearrangements, as discussed earlier (Table 30-1).

Cancer results from multiple and sequential genetic mutations occurring in a somatic cell. At some juncture, a critical mutation occurs, and the cell becomes self-perpetuating or clonal. A *clone* is a cell population derived from a single progenitor.¹⁷ A cytogenetic clone exists if two or more cells contain the same structural abnormality or supernumerary marker chromosome or if three or more cells are missing the same chromosome. The primary aberration or stemline of a clone is a cytogenetic abnormality that is frequently observed as the sole abnormality associated with the cancer. The secondary aberration or sideline includes abnormalities additional to the primary aberration.¹⁷ In chronic myelogenous leukemia, the primary aberration is the Philadelphia chromosome resulting from a translocation between the long arms of chromosomes 9 and 22, $t(9;22)(q34;q11.2)$. A sideline of this clone would include secondary abnormalities, such as trisomy for chromosome 8, written as $+8,t(9;22)(q34;q11.2)$.

Leukemia

Leukemias are clonal proliferations of malignant leukocytes that arise initially in the bone marrow before disseminating to the peripheral blood, lymph nodes, and other organs. They are broadly classified by the type of blood cell giving rise to the clonal proliferation (lymphoid or myeloid) and by the clinical course of the disease (acute or chronic). The four main leukemia categories are acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML), chronic lymphocytic leukemia (CLL), and chronic myelogenous leukemia (CML). The World Health Organization (WHO) classification for myeloid malignancies has categorized AML into seven subtypes: AML with recurrent genetic abnormalities; AML with myelodysplasia-related changes; therapy-related myeloid neoplasms; AML not otherwise specified; myeloid sarcoma; myeloid proliferations related to Down syndrome; and blastic plasmacytoid dendritic cell neoplasm (Chapter 35).²⁰ "AML with recurrent genetic abnormalities" is a classification based on the cytogenetic abnormalities observed (Box 30-1). Some of the divisions of the French-American-British (FAB) classification²¹ are included in the "not otherwise classified" category. The WHO has classified lymphoid leukemias by precursor cell type, B or T (Chapter 35).

BOX 30-1 Acute Myeloid Leukemia (AML) with Recurrent Genetic Abnormalities*

AML with $t(8;21)(q22;q22);RUNX1T1/RUNX1$
 AML with $inv(16)(p13.1q22)$ or $t(16;16)(p13.1;q22);MYH11/CBFB$
 Acute promyelocytic leukemia with $t(15;17)(q24.1;q21.1);PML/RARA$
 AML with $t(9;11)(p22;q23);MLL3/MLL(KMT2A)$
 AML with $t(6;9)(p23;q34);DEK/NUP214$
 AML with $inv(3)(q21q26.2)$ or $t(3;3)(q21.3;q26.2);RPN1/MECOM$
 AML (megakaryoblastic) with $t(1;22)(p13;q13);RBM15/MKL1$
 AML with normal chromosomes and mutated $NPM1(5q35.1)$
 AML with normal chromosomes and mutated $CEBPA(19q13.1)$

Modified from Swerdlow SH, Campo E, Harris NL, et al, editors: *WHO classification of tumours of haematopoietic and lymphoid tissues*, ed 4, Lyon, France, 2008, IARC Press.

*Updated per Hugo Nomenclature Database, May 2013

Chronic Myelogenous Leukemia

The first malignancy to be associated with a specific chromosome defect was CML, in which approximately 95% of patients were found to have the Philadelphia chromosome translocation, $t(9;22)(q34;q11.2)$ by G-banded analysis.^{22,23} The Philadelphia chromosome (derivative chromosome 22) is characterized by a balanced translocation between the long arms of chromosomes 9 and 22. At the molecular level, the gene for *ABL1*, an oncogene on chromosome 9, joins a gene on chromosome 22 named *BCR*. The result of the fusion of these two genes is a new fusion protein of about 210 kD with growth-promoting capabilities that override normal cell regulatory mechanisms (Figures 30-18 and 30-19) (Chapter 33).²⁴

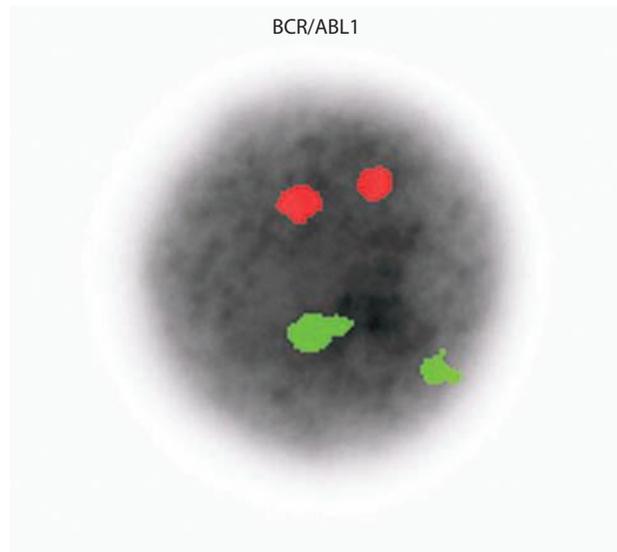


Figure 30-18 Normal bone marrow interphase cell hybridized with the *BCR* (green) and *ABL1* (red) genes (Abbott Molecular, Des Plaines, IL). The two red and two green signals represent the genes on the normal chromosomes 9 and 22. (Courtesy the Cytogenetics Laboratory, Indiana University School of Medicine, Indianapolis, IN.)

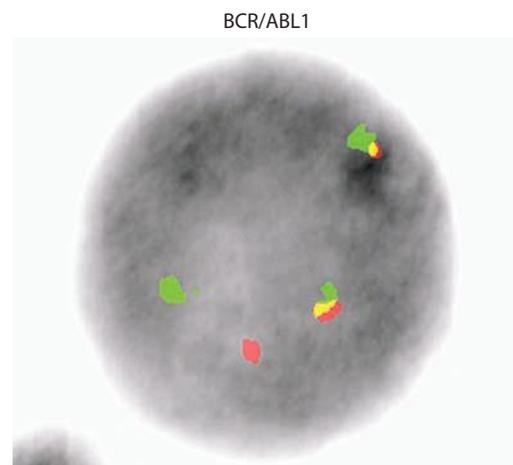


Figure 30-19 Abnormal bone marrow interphase cell with one *BCR* (green) and one *ABL1* (red) signal (Abbott Molecular, Des Plaines, IL) representing the normal chromosomes and two fusion signals from the derivative chromosomes 9 and 22. (Courtesy the Cytogenetics Laboratory, Indiana University School of Medicine, Indianapolis, IN.)

The fusion protein activates tyrosine kinase signaling to drive proliferation of the cell. This signaling can be blocked by imatinib mesylate (Gleevec; Novartis Pharmaceuticals, East Hanover, NJ) or another tyrosine kinase inhibitor.²⁵ Patient response to imatinib is monitored by cytogenetic analysis and FISH. At diagnosis, the characteristic karyotype is the presence of the Philadelphia chromosome in all cells analyzed. After treatment with imatinib for several months, the karyotype typically has a mixture of abnormal and normal cells indicating patient response to therapy. *Complete response* is defined as a bone marrow karyotype with only normal cells. Therapeutic response is often monitored using peripheral blood instead of a bone marrow aspirate. In contrast to the bone marrow, the peripheral blood does not contain spontaneously dividing cells. As a result, chromosomal analysis of a specimen of unstimulated peripheral blood may be unsuccessful because of the absence of dividing cells. In these cases, FISH with probes for the specific abnormality is performed on 200 or more interphase (nondividing) cells of the peripheral blood specimen to search for chromosomally abnormal cells. The detection of cytogenetic abnormalities in interphase (nondividing) cells is an important advantage of FISH technology.

Acute Leukemia

The Philadelphia chromosome is also observed in acute leukemia. It is seen in about 20% of adults with ALL, 2% to 5% of children with ALL, and 1% of patients with AML.²⁶⁻²⁸ In childhood ALL, chromosome number is critical for predicting the severity of the leukemia. Children whose leukemic cells contain more than 50 chromosomes (hyperdiploid karyotype) have the best prognosis for complete recovery with therapy. Recurring translocations observed in ALL include t(4;11)(q21;q23), t(12;21)(p13;q22), and t(1;19)(q23;p13.3). Each translocation is associated with a prognostic outcome and assists oncologists in determining patient therapy. The t(4;11) translocation is the one most commonly found in infants with acute lymphoblastic leukemia. Rearrangements of the *AFF1* gene on chromosome 4 and the *MLL* gene on chromosome 11 occur in this translocation.^{29,30} Disruption of the *MLL* gene is seen in both ALL and AML (Figures 30-20 and 30-21).

The AMLs are subdivided into several morphologic classifications ranging from M0 to M7 according to the FAB classification (Chapter 35).^{31,32} Characteristic chromosome translocations are associated with some subgroups and were incorporated into the WHO classification. Among them is a translocation between the long arms of chromosomes 8 and 21, t(8;21)(q22;q22), which is representative of AML with maturation. Acute promyelocytic leukemia is associated with a translocation between the long arms of chromosomes 15 and 17, t(15;17)(q24;q21) (Figure 30-22). A pericentric inversion of chromosome 16, inv(16)(p13.2q22), is seen in AML with increased eosinophils. The inversion juxtaposes the core the binding factor beta (*CBFB*) gene on 16q with the myosin heavy chain gene (*MYH11*) on 16p to form a new fusion protein (Figure 30-23).³³ These recurring translocations have enabled researchers to localize genes important for cell growth and regulation. As with acute lymphoblastic leukemia, the specific translocation

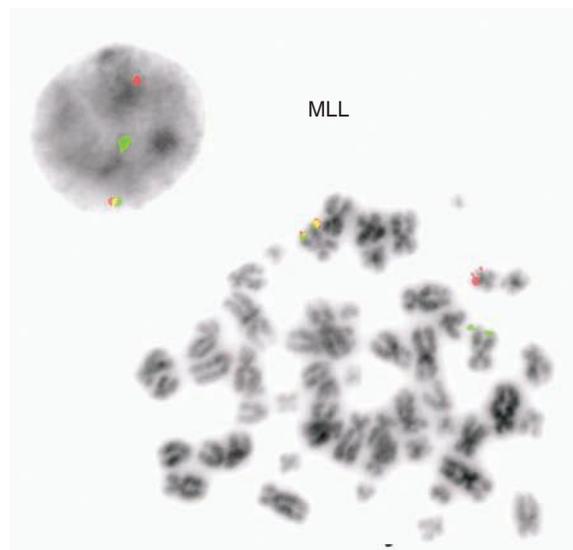


Figure 30-20 Bone marrow metaphase cell with fusion *MLL(KMT2A)* signal on the normal chromosome 11 and split red and green signals on the translocated chromosomes, representing a disruption of the *MLL(KMT2A)* gene. (Courtesy the Cytogenetics Laboratory, Indiana University School of Medicine, Indianapolis, IN.)

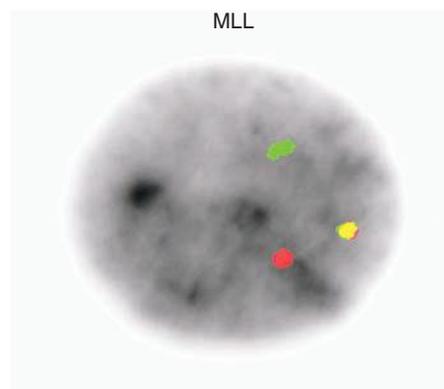


Figure 30-21 Bone marrow interphase cell with a fusion signal (normal chromosome 11) and split red and green signals from the *MLL(KMT2A)* gene representing a rearrangement. (Courtesy the Cytogenetics Laboratory, Indiana University School of Medicine, Indianapolis, IN.)

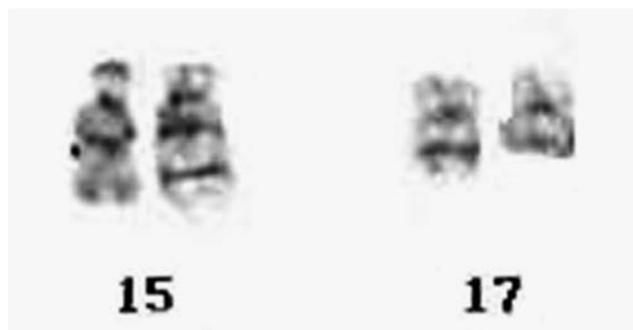


Figure 30-22 Bone marrow metaphase chromosomes 15 and 17 homologues showing a translocation between the long arms of chromosomes 15 and 17, t(15;17)(q24.1;q21.1), diagnostic of acute promyelocytic leukemia. The abnormal chromosomes are on the right. (Courtesy the Cytogenetics Laboratory, Indiana University School of Medicine, Indianapolis, IN.)

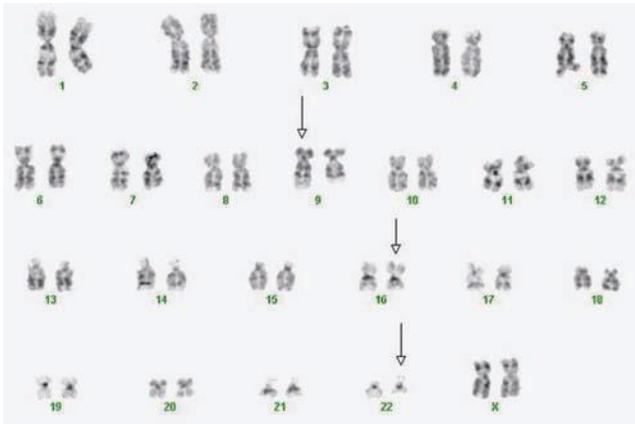


Figure 30-23 Bone marrow karyogram for a patient with acute myeloid leukemia (AML) showing a translocation, $t(9;22)(q34;q11.2)$, and an inverted chromosome 16, $inv(16)(p13.1q22)$. (Courtesy the Cytogenetics Laboratory, Indiana University School of Medicine, Indianapolis, IN.)

in AML often predicts patient prognosis and response to therapy. Understanding the molecular consequences of the cytogenetic mutations, such as the *BCR/ABL1* translocation, provides the fundamental information for the development of targeted therapies.

Solid Tumors

Just as recurring structural and numeric chromosome defects have been observed in the hematologic malignancies, a wide range of nonrandom abnormalities have also been found in solid tumors. Most of these abnormalities confer a proliferative advantage on the malignant cell and serve as useful prognostic indicators. Amplification (increased copy number) of the gene *HER2* (also called *ERBB2*) on chromosome 17, a transmembrane growth factor receptor, is associated with an aggressive form of invasive breast cancer.^{34,35} FISH with probes for the *HER2* gene and an internal control (17 centromere) can determine if there is gene amplification in the tumor (Figure 30-24).³⁶ If FISH testing

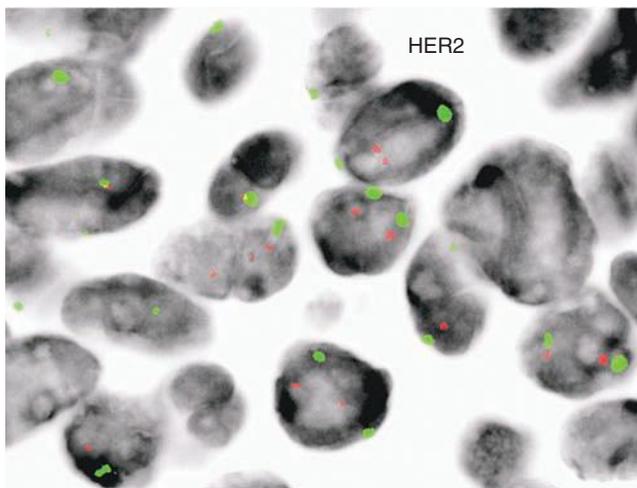


Figure 30-24 Normal interphase nuclei from a paraffin-embedded tissue section hybridized with probes for *HER2* (red) and the chromosome 17 centromere (green) (Abbott Molecular, Des Plaines, IL). Two green and two red signals are seen per cell.

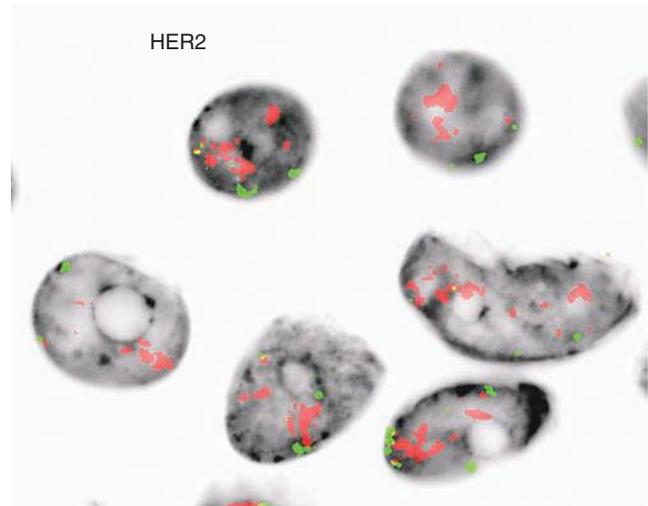


Figure 30-25 Tissue section from a breast cancer demonstrating amplification of *HER2*. The tissue was hybridized with fluorescence in situ hybridization probes for *HER2* (red) and the chromosome 17 centromere probe (green) (Abbott Molecular, Des Plaines, IL). The number of *HER2* signals exceeds the number of centromere signals, which indicates selective amplification of *HER2*.

shows amplification to be present, the patient is eligible for targeted therapy with a monoclonal antibody, trastuzumab (Figure 30-25).³⁷ FISH for *HER2* typically is performed on tissue sections from the paraffin-embedded tumor block.

CHROMOSOMAL MICROARRAY ANALYSIS

Chromosomal microarray (CMA) is a fluorescence-based molecular technique for submicroscopic analysis of genomic DNA. CMA testing increases the detection of clinically significant imbalances over a karyotype.³⁸ CMA is performed utilizing a glass slide or chip platform. Chromosomal microarrays, like standard cytogenetic analysis, look at the entire genome but with higher levels of resolution (base pair or kilobase level) determined by the number and composition of targets on the array. Using a SNP-based array, patient DNA is hybridized to a chip composed of greater than 2 million markers that detect copy number variation (gains and losses) and SNP polymorphisms. Single nucleotide polymorphism (SNP) probes detect position-specific markers that have different forms (polymorphic). Analysis of the SNP data from a specimen allows for detection of copy neutral loss of heterozygosity or uniparental disomy, as well as gains and losses of genomic DNA. Regions of imbalance (copy gain or copy loss) in the patient specimen are assessed relative to a reference control. The yield of detection of abnormalities is increased from an average of 3% to 11% due to the high resolution of the array (Figures 30-26 and 30-27).³⁸⁻⁴⁰ This technique is presently used primarily for diagnosis of constitutional (inherited) disorders, but emerging applications for cancer are in development.

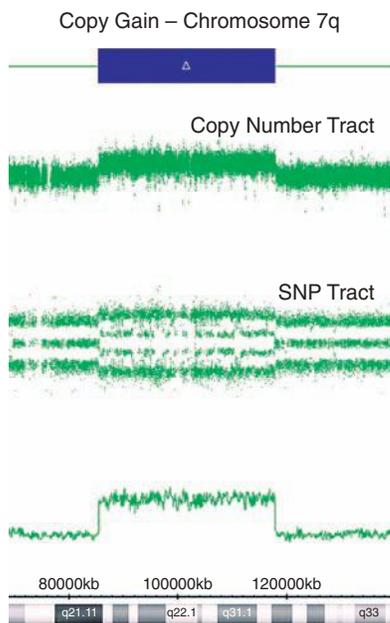


Figure 30-26 Chromosomal microarray diagram demonstrating approximately a 32.4-Mb gain of genetic material between bands 7q21 and 7q31. (Courtesy of the Cytogenetics Laboratory, Indiana University School of Medicine, Indianapolis, IN.)

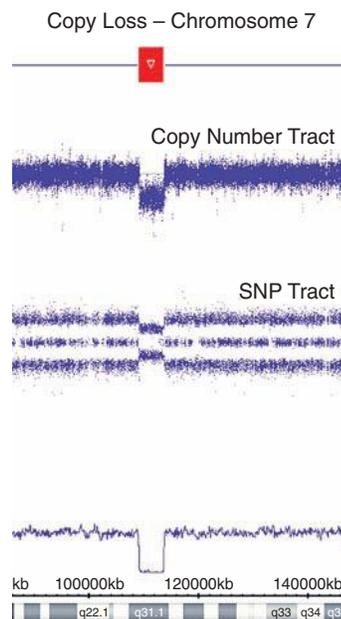


Figure 30-27 Chromosomal microarray diagram demonstrating approximately a 4.6 Mb loss at band 7q31.1. (Courtesy of the Cytogenetics Laboratory, Indiana University School of Medicine, Indianapolis, IN.)

SUMMARY

- Cytogenetics is the study of chromosome structure and inheritance.
- Chromosome disorders are secondary to structural or numeric chromosomal abnormalities involving the rearrangement or the loss or gain of a piece of a chromosome or the entire chromosome.
- Nonrandom chromosome abnormalities are associated with cancer.
- A chromosome is composed of a double helix strand of DNA. Attached to the backbone of deoxyribose are adenine (A), guanine (G), cytosine (C), and thymine (T).
- During mitosis, cells can be chemically treated to arrest cell progression in metaphase so that chromosomes can be analyzed.
- Q-banding differentiates chromosomes into bands of different widths and relative brightness, revealing a banding pattern unique to each individual chromosome.
- Other stains used to identify chromosomes may require pretreatment of the slide for analysis. These include G-banding, C-banding, and AG-NOR-banding.
- FISH, a molecular cytogenetic technique, uses DNA or RNA probes and fluorescence microscopy to identify individual chromosomes and targeted chromosomal loci. Metaphase and interphase cells can be analyzed by FISH.
- Tissues used for chromosome analysis typically include bone marrow cells and peripheral blood lymphocytes, amniotic fluid, nonneoplastic tissue, and tumors.
- A normal cell contains 46 chromosomes, which includes 2 sex chromosomes (XX or XY).
- Defects in chromosomes can be categorized as numeric or structural. Numeric abnormalities can be subclassified as aneuploidy and polyploidy.
- Structural rearrangements include inversions, deletions, isochromosomes, ring formations, insertions, translocations, and duplications.
- Specific structural rearrangements are associated with distinct subtypes of leukemias and may assist in diagnosis, prognosis, and monitoring of therapy. Solid tumors also may be analyzed using cytogenetics.
- Chromosomal microarray testing utilizes a microarray platform to detect abnormalities at a submicroscopic level of resolution. The higher resolution increases the detection of chromosomal abnormalities.

Now that you have completed this chapter, go back and read again the case study at the beginning and respond to the questions presented.

REVIEW QUESTIONS

Answers can be found in the Appendix.

- G*-banding refers to the technique of staining chromosomes:
 - To isolate those in the G group (i.e., chromosomes 21 and 22)
 - In the G₀ or resting stage
 - Using Giemsa stain
 - To emphasize areas high in guanine residues
- Which of the following compounds is used to halt mitosis in metaphase for chromosome analyses?
 - Imatinib
 - Fluorescein
 - Trypsin
 - Colchicine
- One arm of a chromosome has 30 bands. Which band would be nearest the centromere?
 - Band 1
 - Band 15
 - Band 30
- Which of the following is *not* an advantage of the use of FISH?
 - It can be used on nondividing cells.
 - It can be used on paraffin-embedded tissue.
 - It can detect mutations that do not result in abnormal banding patterns.
 - It must be performed on dividing cells.
- Which of the following types of mutations would likely *not* be detectable with cytogenetic banding techniques?
 - Point mutation resulting in a single amino acid substitution
 - Transfer of genetic material from one chromosome to another
 - Loss of genetic material from a chromosome that does not appear on any other chromosome
 - Duplication of a chromosome resulting in 3n of that genetic material
- Which of the following describes a chromosomal deletion?
 - Point mutation resulting in a single amino acid substitution
 - Transfer of genetic material from one chromosome to another
 - Loss of genetic material from a chromosome that does not appear on any other chromosome
 - Duplication of a chromosome resulting in 3n of that genetic material

The chromosome analysis performed on a patient's leukemic cells is reported as 47, XY,+4,del(5)(q31)[20]. Answer questions 7 to 9 based on this description.

- This patient's cells have which of the following mutations?
 - Loss of the entire number 31 chromosome
 - Loss of the entire number 5 chromosome
 - Loss of a portion of the short arm of chromosome 4
 - Loss of a portion of the long arm of chromosome 5
- What other mutation is present in this patient's cells?
 - Polyploidy
 - Tetraploidy
 - An extra chromosome 4
 - Four copies of chromosome 5
- This patient's leukemic cells demonstrate:
 - Structural chromosomal defects only
 - Numeric chromosomal defects only
 - Both structural and numeric chromosomal defects
- Aneuploidy* describes the total chromosome number:
 - That is a multiple of the haploid number
 - That reflects a loss or gain of a single chromosome
 - That is diploid but has a balanced deletion and duplication of whole chromosomes
 - In gametes; *diploid* is the number in somatic cells

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Molecular Diagnostics in Hematopathology

Cynthia L. Jackson and Shashi Mehta*

OUTLINE

Structure and Function of DNA

The Central Dogma: DNA to RNA to Protein

*DNA at the Molecular Level
Transcription and Translation
DNA Replication and the Cell Cycle*

Molecular Diagnostic Testing Overview

Nucleic Acid Isolation

Isolating DNA from Clinical Specimens

Isolating RNA from Clinical Specimens

Amplification of Nucleic Acids by Polymerase Chain Reaction

Polymerase Chain Reaction for Amplifying DNA

Reverse Transcription Polymerase Chain Reaction for Amplifying RNA

Detection of Amplified DNA

Gel Electrophoresis

Restriction Endonuclease Methods

Nucleic Acid Hybridization and Southern Blotting

Cleavage-Based Signal Amplification

DNA Sequencing

Real-Time Polymerase Chain Reaction

Qualitative Real-Time Polymerase Chain Reaction

Quantitative Real-Time Polymerase Chain Reaction

Minimal Residual Disease in Leukemia

Mutation Enrichment Strategies

Chromosome Microarrays Pathogen Detection and Infectious Disease Load Current Developments

OBJECTIVES

After completion of this chapter, the reader will be able to:

1. Describe the structure of DNA, including the composition of a nucleotide, the double helix, and the antiparallel complementary strand orientation.
2. Predict the nucleotide sequence of a complementary strand of DNA or RNA given the nucleotide sequence of a DNA template.
3. Explain the relationship between DNA structure and protein structure.
4. Discuss the process of DNA replication, including replication origin, replication fork, primase, primer, DNA polymerase, Okazaki fragments, leading strand, and lagging strand.
5. Determine the appropriate patient specimen required for DNA isolation to identify an inherited or somatic mutation.
6. Discuss common methods of DNA and RNA isolation.
7. Explain the principle of polymerase chain reaction (PCR), reverse transcriptase PCR, and nucleic acid hybridization.
8. Compare and contrast the methods for detecting target DNA including: gel electrophoresis using intercalating dyes or capillary gel electrophoresis; cleavage-based signal amplification; restriction fragment length polymorphism; and probe hybridization techniques such as Southern blotting.
9. Describe the principle of qualitative real-time PCR and contrast it with endpoint PCR.
10. Discuss the use of quantitative real-time PCR for monitoring minimal residual disease.
11. Explain traditional Sanger DNA sequencing and contrast it with pyrosequencing, and next-generation sequencing (massively parallel sequencing).
12. Describe the use of microarray-based comparative genomic hybridization (aCGH) and single nucleotide polymorphism array (SNP-A) karyotyping for the detection of chromosome copy number alterations.

CASE STUDY**

After studying the material in this chapter, the reader should be able to respond to the following case study:

A 42-year-old man came to the emergency room complaining of pain behind his right knee. He had observed swelling below the knee for the previous 2 days. The patient was in no apparent distress and was experiencing no chest pain, shortness of breath, dyspnea, or hemoptysis. The patient reported no history of trauma except for a right femur break 20 years before. He reported that he was taking no medications and was in good general health. Five years previously, he experienced an episode of deep vein thrombosis (DVT) in his right lower leg and was treated with intravenous heparin followed by oral warfarin (Coumadin) for 3 months. Subsequent to treatment, he experienced occasional pain behind both knees, which he treated with aspirin. He noted that his mother had been diagnosed with carpal tunnel syndrome and had developed DVT, for which she has been taking oral warfarin for 15 years. The patient's job requires frequent long airplane flights. He flies first class and walks around occasionally during the long flights. His leg pain began 1 week after a flight to Europe.

Continued

*The authors acknowledge Dr. Mark E. Lasbury, whose work in prior editions formed the foundation for this chapter.

CASE STUDY**—cont'd

On physical examination, the patient had no evidence of rash or oral ulcers. No petechiae or purpura were noted. He had mild pretibial pitting edema. His right leg measured 36.5 cm at 25 cm distal to the superior aspect of the patella, whereas his left leg measured 33.5 cm in the same location. CBC findings were unremarkable, and both the prothrombin time and activated partial prothrombin time were within the reference intervals. Doppler ultrasonography revealed complete occlusion of the distal superficial femoral vein, anterior tibial vein, and popliteal vein. The diagnosis was DVT without pulmonary emboli. The patient was hospitalized, and a heparin drip was started. The hematologist ordered a factor V (*F5*) Leiden mutation analysis on blood drawn in an EDTA tube. Figure 31-1 illustrates the results of the mutation analysis.

1. What type of specimen is appropriate when analyzing DNA for a hereditary mutation?
2. Examine the gel electrophoresis result (Figure 31-1). Are the correct controls present?
3. What band sizes (in base pairs or bp) appear in the patient's sample?
4. What band sizes are expected for an individual who is homozygous for the factor V (*F5*) Leiden mutation, heterozygous for the mutation, and free of the mutation?
5. Does this patient have the factor V (*F5*) Leiden gene mutation?

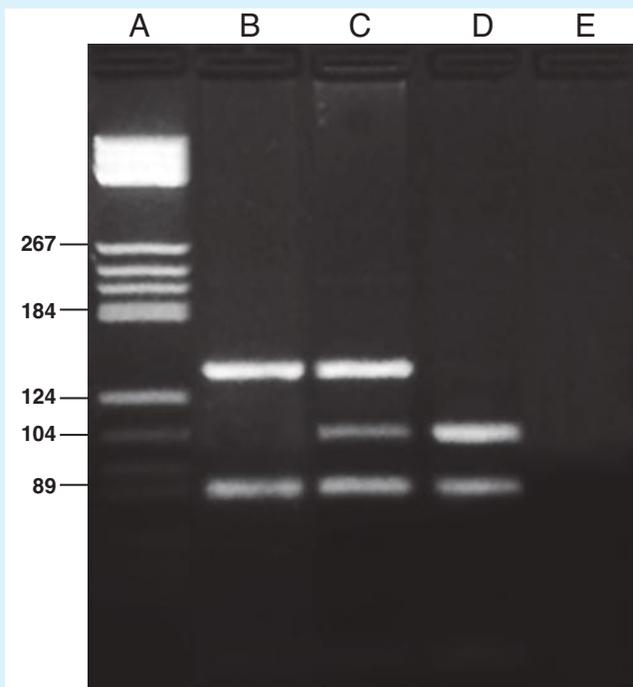


Figure 31-1 Results of the factor V (*F5*) Leiden mutation test on the patient in the case study. Lane A, molecular size marker; B, positive control (homozygous for factor V Leiden mutation); C, patient's sample; D, negative control (no mutation); E, no-DNA control. The expected banding pattern on an agarose gel for the factor V (*F5*) Leiden mutation test is as follows: homozygous for the mutation, 141 and 82 bp bands; heterozygous for the mutation, 141, 104, and 82 bp bands; no mutation (normal or wild-type), 104 and 82 bp bands. A band at 37 bp is barely visible and is difficult to detect on an agarose gel. However, this band is not essential for interpreting the results.

**This case was provided by George A. Fritsma, MS, MLS, manager, The Fritsma Factor: Your Interactive Hemostasis Resource, <http://www.fritsmafactor.com>, sponsored by Precision BioLogic, Inc., Cambridge, Nova Scotia.

Molecular biology techniques enhance the diagnostic team's ability to predict or identify an increasing number of diseases in the clinical laboratory. Molecular techniques also enable clinicians to monitor disease progression during treatment, make accurate prognoses, and predict the response to therapeutics. The short interval required to perform molecular diagnostic tests and analyze their results is an additional positive aspect of this type of testing, resulting in more efficient patient management, especially in cases of infection. The five main areas of hematopathologic molecular testing include detection of mutations, gene rearrangements, and chromosomal abnormalities for diagnosis and prognosis of hematologic malignancies (Box 31-1); detection and quantification of minimal residual disease to monitor treatment of hematologic malignancies; detection of mutations in inherited hematologic disorders (Box 31-2); pharmacogenetic testing to detect

genetic variation affecting certain drug therapies (Box 31-3); and identification of hematologically important infectious diseases (Box 31-4).

STRUCTURE AND FUNCTION OF DNA

The Central Dogma: DNA to RNA to Protein

Much of the stored information needed to carry out cell processes resides in deoxyribonucleic acid (DNA); therefore, proper cellular storage, maintenance, and replication of DNA are necessary to ensure homeostasis. Because molecular testing takes advantage of DNA structure and replication, a review of molecular biology is helpful.

The *central dogma* in genetics is that information stored in the DNA is *replicated* to daughter DNA, *transcribed* to messenger ribonucleic acid (mRNA), and *translated* into a functional protein (Figure 31-2). This process is essential to carry out cellular

BOX 31-1 Major Hematologic Malignancies in Which Molecular Methods Are Performed for Diagnosis and Monitoring Minimal Residual Disease

For Diagnosis:

Acute leukemias
 Myeloid
 Lymphoblastic
 Myeloproliferative neoplasms
 Chronic myelogenous leukemia
 Polycythemia vera
 Essential thrombocythemia
 Primary myelofibrosis
 Myelodysplastic syndromes
 Mature lymphoid neoplasms
 Chronic lymphocytic leukemia
 Lymphomas

For Monitoring Minimal Residual Disease:

Acute leukemias
 Quantification of fusion mRNA transcripts due to translocations
 Quantification of specific B and T cell receptor rearrangements
 Chronic myelogenous leukemia
 Quantification of fusion mRNA transcripts due to translocation

BOX 31-2 Inherited Hematologic Disorders Detected by Molecular Methods

Erythrocyte disorders
 Hemoglobinopathies/thalassemias
 Membrane abnormalities
 Enzyme deficiencies
 Erythropoietic porphyrias
 Leukocyte disorders
 Quantitative disorders
 Functional disorders
 Storage disorders
 Platelet disorders
 Quantitative disorders
 Functional disorders
 Bone marrow failure syndromes
 Coagulopathies
 Thrombophilia

BOX 31-3 Pharmacogenetic Testing for Genetic Variation Affecting Therapy

Warfarin sensitivity
 Cytochrome P450 2C9 variants, CYP2C9*2, CYP2C9*3
 VKORC1 variants
 Clopidogrel sensitivity
 Cytochrome P450 2C19 variants, CYP2C19*17, others
 Thiopurine sensitivity
 Thiopurine S-methyltransferase, TPMT*2, TPMT*3C, TPMT*3A
 Imatinib resistance
 ABL1 mutation analysis

BOX 31-4 Hematologically Important Pathogens Detected by Molecular Methods

Parasitic pathogens

Plasmodium
Filaria
Babesia
Leishmania
Trypanosoma

Fungal pathogens

Bacterial pathogens

Viral pathogens

Parvovirus B19
 Cytomegalovirus
 Epstein-Barr virus
 Human immunodeficiency virus types 1 and 2
 Human T-cell lymphotropic virus type 1

Modified from Paessler M, Bagg A: Use of molecular techniques in the analysis of hematologic diseases. In Hoffman R, Benz EJ Jr, Shattil SJ, et al, editors: *Hematology: basic principles and practice*, ed 4, Philadelphia, 2005, Churchill Livingstone, pp. 2713-2726.

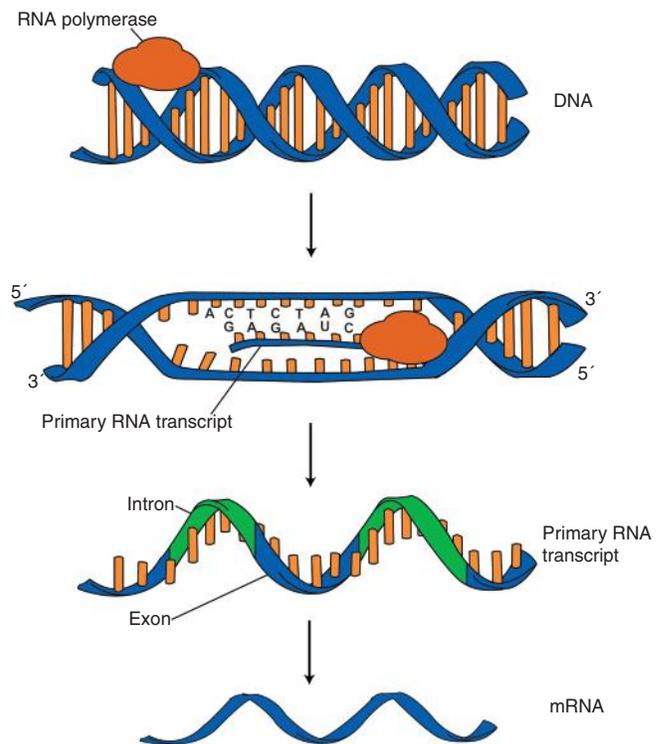


Figure 31-2 RNA polymerase binds to a sequence of DNA called the *promoter region*, which causes the DNA strands to separate. Using one of the DNA strands as a template, RNA polymerase moves along and simultaneously reads the DNA strand, forming the primary messenger RNA (mRNA) transcript by joining the complementary ribonucleotides. The primary mRNA transcript consists of sequences called exons that provide coding information and introns that are excised from the mature mRNA. The spliced mRNA then leaves the nucleus and enters the cytoplasm of the cell where the ribosomes translate the mRNA into protein.

functions while preserving a record of the stored information. In eukaryotes, the initial DNA sequence is composed of *translated* exons separated by *untranslated* introns. The introns are enzymatically excised following transcription from DNA to RNA, and the mature mRNA sequence is then translated. Translation is an enzymatic process wherein mRNA three-nucleotide base sequences called *codons* drive the addition of individual amino acids to the growing peptide. The mature protein then carries out its cellular function, which may be structural or may involve recognition, regulation, or enzymatic activity.

The structural units that carry DNA's message are called *genes*. The human β -globin gene, part of the hemoglobin molecule, provides a good example of replication and transcription, because it was one of the first sequenced and demonstrates the result of aberrant sequence maintenance. A normal (or *wild-type*) β -globin gene contains a sequence of bases that code for a β -globin peptide of 146 amino acids (Chapter 10). One inherited mutation changes a single DNA base. This is called a *point mutation*. The mutation occurs in the portion of the sequence that codes for the sixth amino acid of β -globin. The mutation substitutes the amino acid *valine* for *glutamic acid* in the growing peptide. Valine modifies the overall charge, producing a protein that polymerizes in a low-oxygen environment. This leads to sickled erythrocytes, circulatory ischemia, and poor oxygen exchange between blood and tissues.^{1,2} A mutation in one of the two copies (*alleles*) of this gene inherited from the parents results in a heterozygous condition, or sickle cell trait. In a *heterozygote*, the symptoms of the disease are often unseen or are present only during times of physical stress. If both alleles are mutated, there is overt *homozygous* sickle cell disease, and the symptoms are severe.

Every active gene is translated. Human somatic cells contain 20,000 to 25,000 genes in 2 meters of DNA.^{3,4} Significant packing (Figure 30-3) takes place to reduce the volume of the nucleic acid to the size of chromosomes.

DNA at the Molecular Level

DNA is a duplex molecule composed of two complementary hydrogen-bonded *nucleotide* strands (Figure 31-3). Deoxyribo-

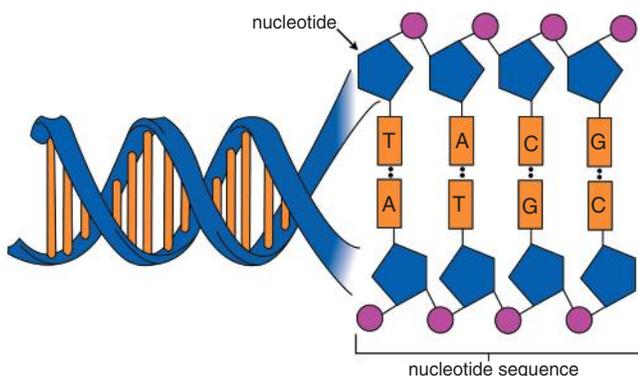


Figure 31-3 DNA is a double-stranded helical macromolecule consisting of nucleotide subunits joined in sequence by deoxyribose molecules (pentagons) and phosphate radicals (circles). The bases thymine (T), adenine (A), cytosine (C), and guanine (G) are illustrated in their standard pairs: thymine to adenine, cytosine to guanine.

nucleotides and ribonucleotides are the building blocks of DNA and RNA, respectively. Each nucleotide is composed of a 5-carbon sugar (pentose), a nitrogenous base, and a phosphate group. The numbers one prime (1') to five prime (5') designate the pentose's carbons. In DNA, the pentose is a ribose in which the hydroxyl group on the 2' carbon is replaced by a hydrogen molecule, hence 2'-deoxyribose (Figure 31-4, A). In RNA, the 2' ribose retains the 2' hydroxyl group. The hydroxyl group present on the 3' carbon of the sugar is crucial for polymerization of the nucleotide monomers to form the nucleic acid strand.

The nitrogenous base is linked to the sugar by a glycosidic bond at the 1' carbon. Four different bases form DNA, but the linkage to the sugar is the same for each. The phosphate group is linked to the sugar at the 5' carbon by a phosphodiester bond (Figure 31-4, B, C). The phosphate group is also crucial

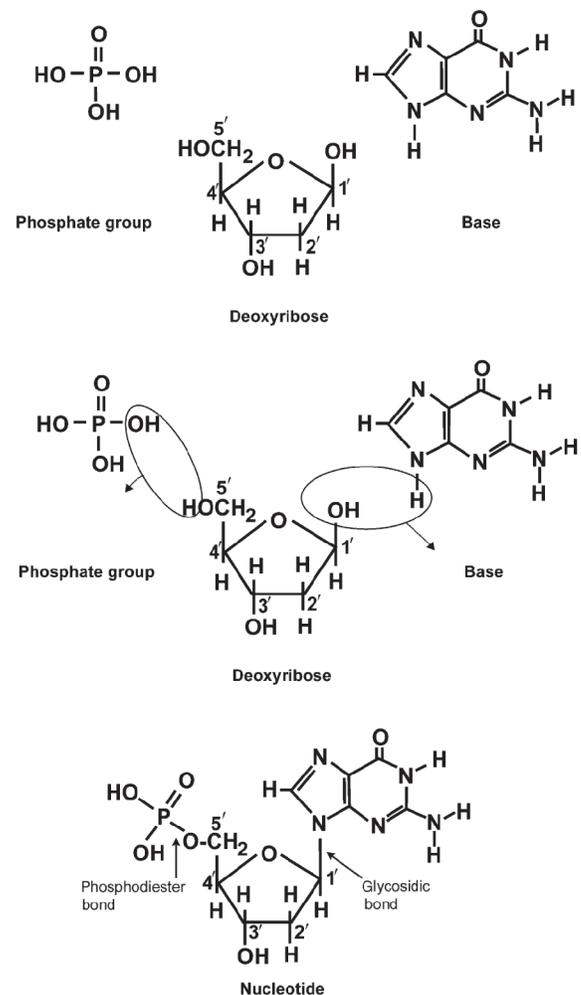


Figure 31-4 A, The pentose sugar, deoxyribose, a phosphate group, and a nitrogenous base compose a DNA nucleotide. The carbons of the deoxyribose molecule are numbered 1' through 5'. The hydroxyl group on the 2' carbon of ribose is replaced by a hydrogen molecule, making the structure a deoxyribose. B, A nucleotide results from the formation of a glycosidic bond between the nitrogenous base and the hydroxyl group on the 1' carbon of deoxyribose and a phosphodiester bond between the phosphate group and the hydroxyl group on the 5' carbon of deoxyribose. C, A nucleotide illustrating the glycosidic and phosphodiester bonds.

for addition of nucleotides to the growing polymer. A sugar, whether ribose or deoxyribose, linked to a nitrogenous base but without a phosphate group, is called a *nucleoside*. A nucleoside cannot be incorporated into DNA, and neither can a nucleotide consisting of only one phosphate group (deoxynucleotide monophosphate, or dNMP). To be incorporated into a growing strand of DNA, the nucleotide must have three phosphate groups linked to one another, referred to as the α -, β -, and γ -phosphates with the α -phosphate linked to the sugar (Figure 31-5).

Creation of a phosphodiester bond between the 3' hydroxyl group of the existing strand and the 5' α -phosphate of the nucleotide monomer requires the enzyme *DNA polymerase*. This enzyme recognizes the hydroxyl group on the 3' carbon of the sugar and bonds the 3' hydroxyl group of one nucleotide with the α -phosphate group of another (Figure 31-5). Polymerization of subsequent nucleotides forms a DNA strand.

DNA consists of two strands that are *antiparallel* and *complementary* (Figure 31-6). One strand begins with a phosphate group attached to the 5' carbon of the first nucleotide and ends with the hydroxyl group on the 3' carbon of the last nucleotide. This strand is in the 5'-to-3' direction. The other strand runs in the 3'-to-5' direction, or antiparallel. The nucleotide sequences composing these strands provide the encoded messages of our genes. Therefore, the addition of nucleotides is highly regulated.

One regulation mechanism arises from the complementary characteristic of the nucleotides. A nucleotide's identity depends on the type of nitrogenous base present on the template. There are two categories of nitrogenous bases in nucleic acids: *purines* and *pyrimidines* (Figure 31-7). The bases *adenine* (A) and *guanine* (G) are double-ringed purines, whereas *thymine* (T) and *cytosine* (C) are single-ringed pyrimidines. Adenine forms hydrogen bonds at two points with thymine (A:T), whereas guanine forms hydrogen bonds at three points with cytosine (G:C). If a strand has a 5'-CTAG-3' sequence, the complementary nucleotides on the 3'-to-5' strand are 3'-GATC-5'. In RNA, the pyrimidine *uracil* (U) takes the place of thymine and forms

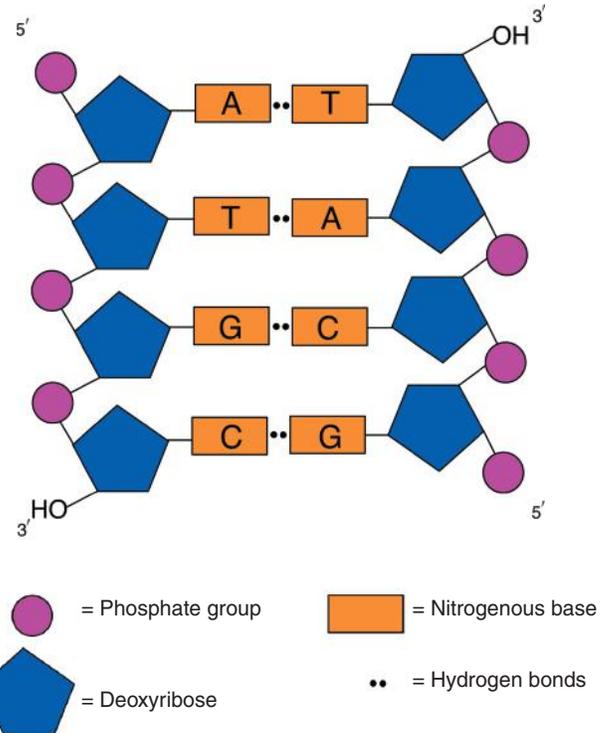


Figure 31-6 DNA consists of two antiparallel and complementary strands. One strand begins with a 5' phosphate group and ends with a 3' hydroxyl group. This strand is read in the 5'-to-3' direction. The other strand begins with a 3' hydroxyl group and ends with a 5' phosphate group. This strand is shown in the 3'-to-5' orientation.

hydrogen bonds with adenine. Hydrogen bonds between A:T and G:C hold the strands together (Figure 31-8). RNA is most often single-stranded but can have significant secondary structure.

In addition to conferring identity to the nucleotide, the nitrogenous bases assist in maintaining a constant width between the strands of a DNA molecule. DNA resembles a ladder, with the repeating sugar and phosphate groups forming the

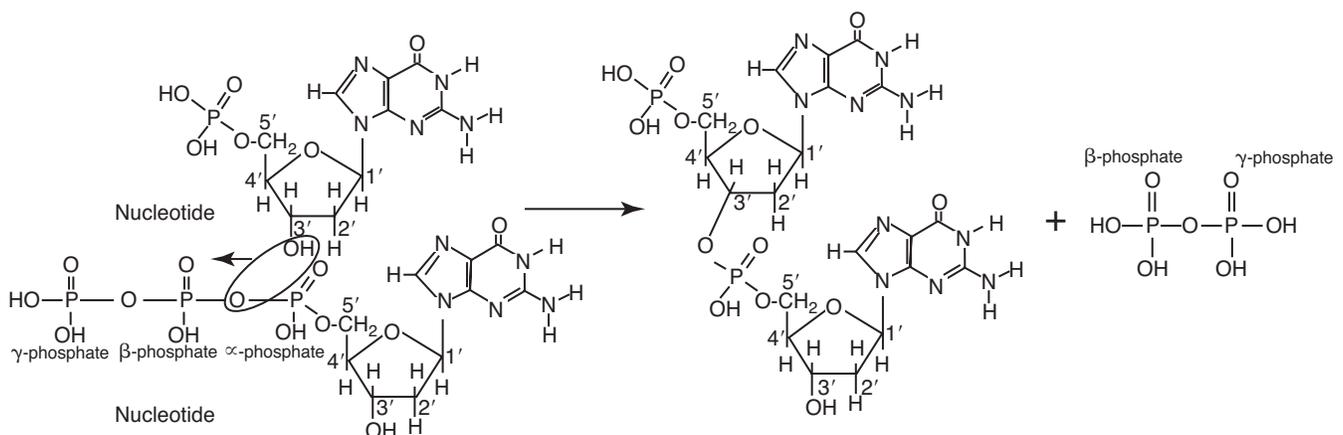


Figure 31-5 The enzyme *DNA polymerase* catalyzes the reaction between the hydroxyl group on the 3' carbon of one nucleotide with the phosphate group bound to the 5' carbon of the downstream nucleotide. The α -phosphate group is split by the 3'-OH, with release of the β - and γ -phosphates.

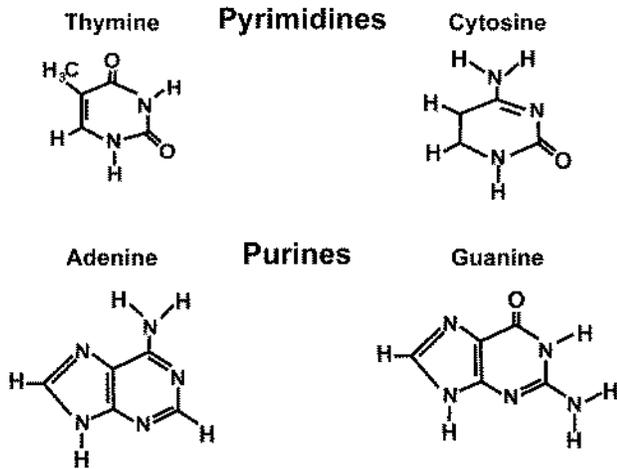


Figure 31-7 The single-ringed pyrimidines (thymine and cytosine) and the double-ringed purines (adenine and guanine) are the code-carrying nitrogenous bases of DNA.

sides of the ladder and the bases forming the rungs. The pairing of a double-ringed purine on one strand with a single-ringed pyrimidine on the other maintains a consistent distance between the DNA strands. This makes DNA flexible, which allows the molecule to twist into a helix. Twisting stabilizes the molecule and protects the bases from their environment.

Transcription and Translation

DNA provides a permanent set of instructions. The cellular enzyme *RNA polymerase* transcribes the code. RNA polymerase recognizes starter sequences called *promoters*. Promoters lie upstream of coding sequences and bind RNA polymerase which separates the DNA strands. The enzyme then slides along the 3' to 5' template DNA strand, "reading" the code and polymerizing (assembling) the complementary ribonucleotides. As the complementary ribonucleotides form hydrogen bonds with the bases of the exposed DNA strand, the RNA polymerase creates phosphodiester bonds to extend the single-stranded primary RNA transcript (Figure 31-2). If the nucleo-

tide sequence of the template DNA strand is 3'-CTAG-5', the primary RNA transcript is 5'-GAUC-3', where uracil is substituted for thymine.

Primary mRNA transcripts are composed of *introns* and *exons*. Introns are untranslated intervening sequences located between the coding portions of genes. Their functions remain unclear, although they may play a role in regulation of gene expression.⁵ Exons are the sequences that encode the gene product. Before mRNA can serve as a translation template, the introns must be excised from the primary transcript and the exons adjoined. The mature mRNA is completed by the addition of a 5' cap and a tail of many repeated adenine nucleotides (polyA tail).⁶ The mature mRNA leaves the nucleus and enters cytoplasm to be translated by the ribosomes.

Ribosomes translate the mRNA code into a peptide sequence. Complexes of proteins and structural ribosomal RNAs (rRNAs) form both large and small ribosome subunits. Mature cytoplasmic mRNA is bound by the small ribosomal subunit at the translation start site. At this point, another series of elements is introduced, *transfer RNAs* (tRNAs), each bound to its specific amino acid. Because there are 20 natural amino acids, there are 20 tRNAs. Each tRNA has a specific nucleic acid sequence located at the point of interaction with the mRNA, complementary to the nucleotide sequence of the mRNA. Each tRNA interacting sequence (anticodon) complements a specific three-nucleotide sequence (codon) of the mRNA.

The mRNA codon AUG is the most common translation start site and codes for the amino acid methionine. The first step in translation is hydrogen bonding of the appropriately charged tRNA (with a bound methionine) to the start codon of the mRNA. The appropriate tRNA is then bonded to the adjacent codon, and a peptide bond is catalyzed between the two amino acids. The peptide bond forms between the carboxyl terminus of the methionine in the existing peptide chain and the amino terminus of the amino acid to be added. Hydrogen bonding of tRNAs to the codons and the formation of the peptide bonds are mediated by the ribosome. With addition of more amino acids, translation proceeds until a termination codon is reached. Three termination codons exist that do not

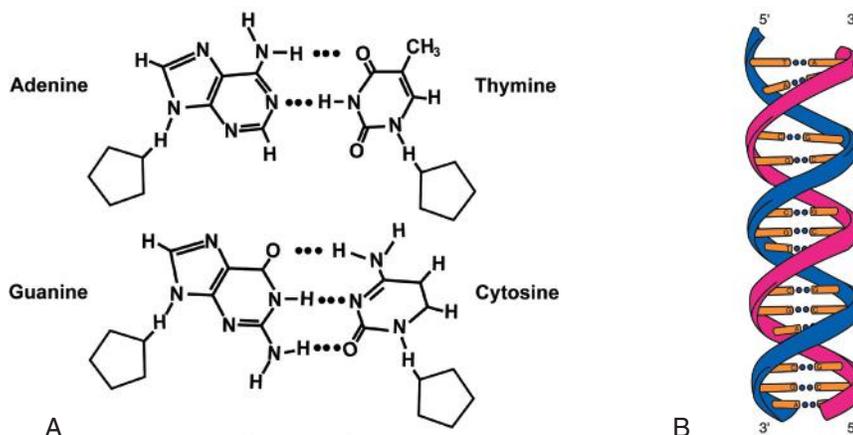


Figure 31-8 A, The purine adenine forms two hydrogen bonds with the pyrimidine thymine. The purine guanine forms three hydrogen bonds with the pyrimidine cytosine. B, The two strands maintain a consistent distance from each other, which allows DNA to twist into a helix.

code for any amino acid and terminate translation: UAA, UAG, and UGA. The ribosome then dissociates, and the peptide folds to its functional shape.

DNA Replication and the Cell Cycle

After cells carry out their functions, they either divide via mitosis or die via *apoptosis*, also called *programmed cell death* (Chapter 6). The cell cycle progresses through a defined sequence (Figure 31-9). *Interphase* is made up of the G_1 , S, and G_2 phases. During the G_1 phase, the cell grows rapidly and performs its cellular functions. S phase is the synthesis stage, in which DNA is replicated. The G_2 phase is the period when the cell produces materials essential for cell division. The *M phase* refers to mitosis, during which two identical daughter cells are produced, each of which receives one entire set of the DNA that was replicated during S phase. Checkpoints occur at the end of G_1 before DNA replication in the S phase, and at the end of G_2 before mitosis in the M phase. The checkpoints have complex mechanisms to stop the progression of the cell cycle if a problem is detected, at which point the cell will undergo apoptosis. Some cells exit the cell cycle during the G_1 phase and enter a phase called G_0 . Cells in G_0 normally do not reenter the cell cycle and remain alive performing their function until apoptosis occurs.

DNA replication during the S phase requires a complex orchestration of events; this discussion focuses on those events that are exploited for molecular diagnostic testing. Contained within the double-stranded DNA helix are multiple *origins of replication*. At each origin, the enzyme *helicase* disrupts the hydrogen bonds, and untwists and separates the DNA strands producing two *replication forks*. Here a deoxyribonucleotide (deoxynucleotide triphosphate, or dNTP) polymerizes to form new complementary strands (Figure 31-10). DNA replication occurs bidirectionally from the two replication origin sites. Each DNA strand in the replication fork serves as a template for the formation of a daughter or complementary strand through the activity of DNA polymerase.⁷ The DNA polymerase substrate is the *free hydroxyl group* located on the 3' carbon of a deoxyribonucleotide. DNA polymerase recognizes this group

and catalyzes the joining of the complementary deoxyribonucleotide. DNA polymerase reads the DNA template in the 3'-to-5' direction, and the complementary strand is synthesized in the 5'-to-3' direction.

A *primer* is required to provide the free 3' hydroxyl group that is necessary for DNA polymerase activity. The enzyme *primase* synthesizes short RNA polymers complementary to the template that serve as primers to initiate DNA synthesis. At the replication origin, the primer hybridizes to the 3' end of the 5'-to-3' (top) template strand (Figure 31-10). Then DNA polymerase recognizes the free hydroxyl group on the 3' carbon of the last nucleotide in the primer and catalyzes the formation of phosphodiester bonds between the correct complementary nucleotide triphosphate and the primer, releasing the β - and γ -phosphate groups. DNA polymerase continues adding deoxyribonucleotides along the replication fork, going to the left of the replication origin, producing the complementary strand called the *leading strand*.

The second template strand, called the *lagging strand*, is also read in the 3'-to-5' direction. To form a complementary strand, a primer hybridizes to the exposed 3' end of the replication fork. To proceed in the 5'-to-3' direction, nucleotides are added in fragments toward the origin of replication. As the left replication fork extends to open more of the template strands for replication, additional primers are hybridized, and DNA polymerase uses the primers to initiate the formation of the complementary strand, continuing until it meets a previously hybridized primer.

DNA polymerase not only joins nucleotides, but it also degrades the RNA primers and fills in the correct complementary deoxyribonucleotides. Because the replication of the lagging strand produces many small fragments, it is called *discontinuous replication*, and the fragments are called *Okazaki fragments*. Finally, the enzyme *ligase* joins the discontinuous fragments. The replication fork to the right (downstream) is replicated in the same fashion, although the lagging strand is now formed complementary to the top (5'-to-3') strand, and the leading strand is formed from the 3'-to-5' strand; the opposite of the situation described occurs for the left replication fork (Figure 31-10).

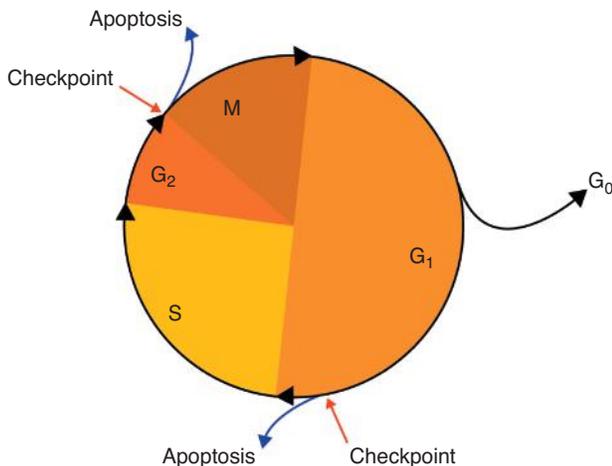


Figure 31-9 The cell cycle consists of interphase and mitosis. Interphase is divided into G_1 , S, and G_2 phases. Cell growth occurs during G_1 . During the S phase, DNA synthesis or replication occurs. The cell prepares for mitosis during the G_2 phase. During mitosis the cell divides, producing two identical daughter cells. The cells may also enter a quiescent phase called G_0 , where the cell functions but does not divide. There are two critical times in the cell cycle that are checkpoints where the cell will either continue through the cell cycle or undergo apoptosis. The first checkpoint is before S phase and DNA replication, and the second checkpoint is at the end of G_2 , where the cell will either enter mitosis or undergo apoptosis.

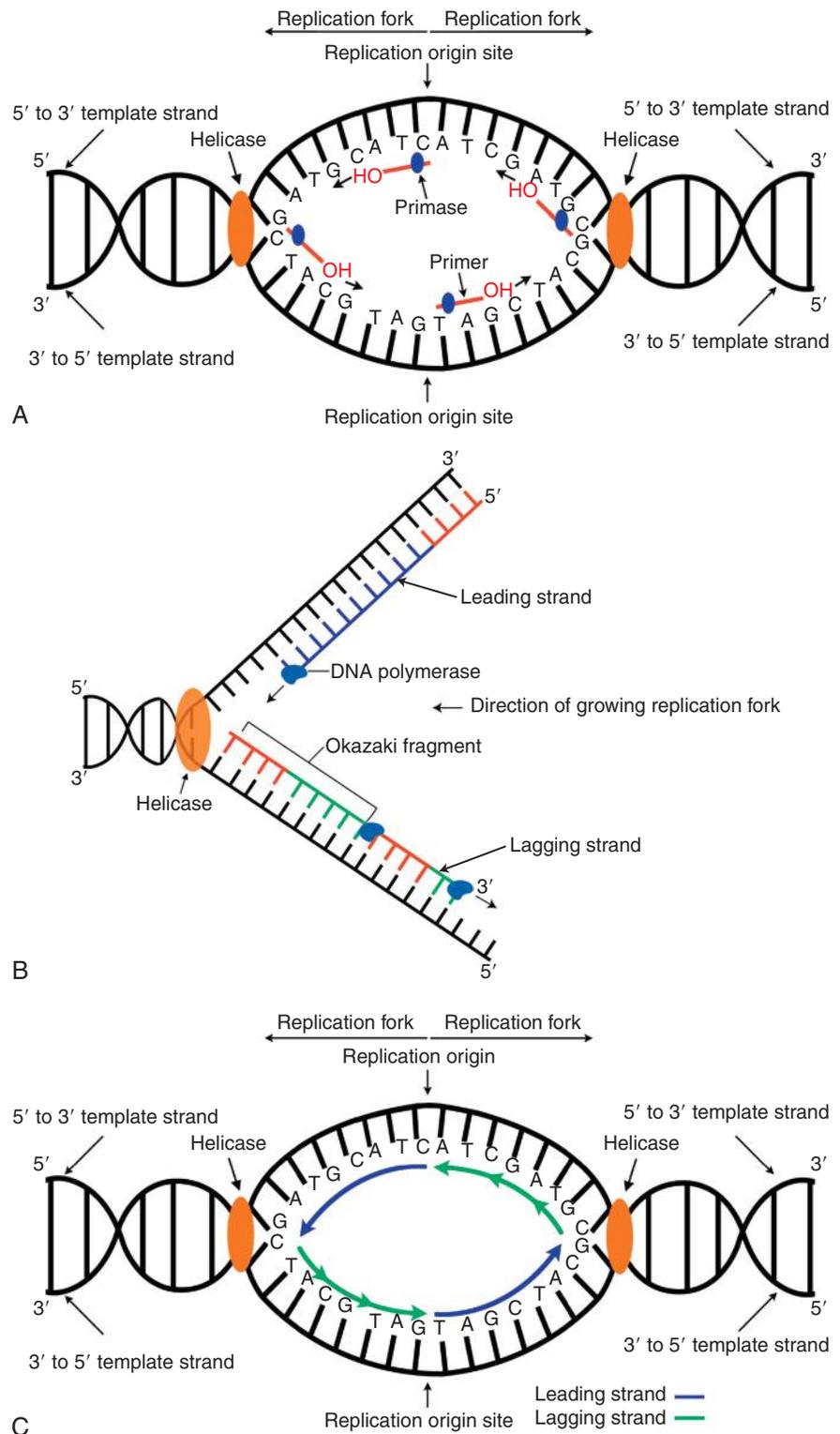


Figure 31-10 DNA Replication. **A**, Primases synthesize RNA primers that anneal to the single-stranded template strands. The primers must be oriented in such a way that the hydroxyl group on the 3' end of the primers is available for deoxyribonucleotide addition by DNA polymerase. **B**, DNA polymerase extends the primer located on the 5'-to-3' coding strand, producing the complementary leading strand (*blue*). On the 3'-to-5' template strand, DNA polymerase extends the primers, producing Okazaki fragments. The primer ribonucleotides (*red*) are replaced with deoxynucleotides by DNA polymerase to produce the complementary lagging strand (*green*). **C**, Bidirectional DNA replication shown in which the 5'-to-3' parent strand serves as the template for producing the continuous leading strands on a replication fork to the left of an origin. The 3'-to-5' parent strand is the template for the lagging strands, which are produced in a discontinuous manner. The continuous and discontinuous strands are reversed on the replication fork to the right.

The cell cycle is highly regulated. At certain critical points within the cycle, decisions are made to continue or begin cell death via apoptosis. This decision may depend on the state of the DNA replicated. Normally, the cell detects errors made during replication and either corrects them or begins apoptosis. This prevents the persistence of daughter cells with genetic

errors. If the sensing molecules fail, cell division may continue. Debilitating mutations that mediate cell cycle control may result in tumor formation. In summary, DNA synthesis and accurate cell cycle control demand that the integrity of the nucleotide sequence be maintained during DNA replication.

MOLECULAR DIAGNOSTIC TESTING OVERVIEW

DNA or RNA sequences are used to diagnose and monitor solid tumors, acute leukemia, myeloproliferative disorders, myelodysplastic neoplasms, inherited thrombosis risk factors, and viral, parasitic, and bacterial infections. Molecular diagnostic testing exploits the enzymes and processes of DNA replication. Most molecular testing methods use replication—for example, polymerase chain reaction (PCR)—to make millions of *amplicons* (copies) of a DNA sequence of interest. Further, creation of synthetic DNA requires the use of short sequences used as either *primers* or *probes* to locate specific DNA or RNA sequences within vast populations of nucleic acids.

Specific mutations are associated with hematologic disease. These are detected by allele-specific amplification methods, DNA sequencing, or restriction fragment length polymorphism analysis of amplified material. Messenger and ribosomal RNA also may be amplified through a process called *reverse transcriptase PCR* (RT-PCR). Using mRNA, the existence of mutations that are being actively translated can be detected. Assessment of mRNA shows whether a mutation is expressed in a certain cell type or tissue and can be used to quantitatively determine the level of transcription of a gene. It can also be used to detect and monitor chromosome translocations that produce novel chimeric mRNA transcripts in conditions where the breakpoints are too widely separated to be detected by PCR amplification of DNA.

Most molecular tests use DNA amplification such as PCR, generating multiple amplicons of the target sequence. Amplification is meant to be specific to the sequence of interest in the sample being tested; however, it will amplify any DNA that is present in the reaction. Consequently, it is critical to eliminate contamination of newly isolated target DNA with amplicons from previously amplified samples. Contamination can be avoided by designating separate laboratory locations for each step, having a unidirectional work flow, and employing appropriate controls. Operators routinely employ ultraviolet (UV) light and bleach to induce strand breaks in contaminating DNA on work surfaces and a *uracil-N-glycosylase* system that destroys previously amplified DNA can also be incorporated into the PCR reactions.

In genetically based hematologic disease, mutations and polymorphisms can occur that do not affect function. Individuals vary in genetic sequences coding for identical proteins. Such single nucleotide polymorphisms are commonly detected but might not be associated with disease. With these caveats in mind, several techniques are presented and an example from hematopathology is given for each. [Box 31-5](#) is a summary of molecular methods with hematopathology applications.

NUCLEIC ACID ISOLATION

Isolating DNA from Clinical Specimens

Most molecular diagnostic tests begin with the isolation of DNA or RNA from a patient specimen. To test for a mutation

BOX 31-5 Molecular Methods with Hematopathology Applications

- Nucleic acid isolation
 - DNA
 - RNA
- Amplification of nucleic acid
 - Polymerase chain reaction (PCR)
 - Reverse transcriptase PCR
- Detection of amplified DNA
 - Electrophoresis
 - Restriction endonuclease methods
 - Nucleic acid hybridization and Southern blotting
 - Cleavage-based signal amplification
 - DNA sequencing
- Real-time PCR
 - Qualitative
 - Quantitative
 - Minimal residual disease in leukemia
 - Mutation enrichment strategies
- Chromosomal microarrays
- Pathogen detection and infectious disease load
- Current developments
 - Mass spectrometry
 - Digital PCR
 - Next-generation sequencing

in patient DNA, the patient's DNA is isolated. To test for microorganism DNA, as in an infection, DNA is also isolated from the patient specimen because it will include the organism DNA. The preferred nucleic acid for clinical diagnosis is DNA because it is inherently more stable than RNA and is less labor intensive to isolate.

The molecular laboratory isolates nucleic acid from a wide variety of clinical specimen types. Patient specimens for human DNA isolation may include peripheral blood, bone marrow, tissue biopsy specimens (both fresh and formalin fixed paraffin-embedded), needle aspirates, body fluids, saliva, and cheek swabs. Blood, saliva, or cheek swab specimens are all appropriate for identifying an inherited defect, although blood is the most common specimen type. Every nucleated cell contains a full complement of DNA. If individuals inherit a mutation, it is present in the DNA of all their nucleated cells, both gamete and non-gamete (*somatic*) cells. Thus the DNA in the nucleus of white blood cells can reveal inherited mutations. In solid tumors, *somatic* (acquired) *mutations* are detected by analyzing DNA from the suspect tissue. For identification of infectious disease organisms by molecular techniques, DNA must also be isolated from the affected tissues. Peripheral blood is adequate for infections with viruses such as human immunodeficiency virus (HIV) and cytomegalovirus (CMV) that infect blood cells, whereas cerebrospinal fluid is required for meningeal infections.

Whole blood is preferentially collected in an ethylenediaminetetraacetic acid (EDTA) tube to prevent clotting and to inhibit enzymes that may digest DNA, although other tubes

may also be acceptable. The red blood cells (RBCs) are removed by taking advantage of the differential lysis in hypotonic buffer due to differing osmotic fragility between white and red blood cells. Incubation in hypotonic buffer will result in the red blood cells lysing before the white blood cells, thus allowing the WBCs to be removed from the hemoglobin and lysed RBCs by centrifugation. Hemoglobin is a potent inhibitor of PCR and other downstream procedures.⁸

DNA from tissue suspected of being cancerous can be isolated from formalin-fixed, paraffin-embedded tissue sections mounted on glass microscope slides or whole sections cut directly into a microfuge tube. Tissue is obtained from the entire section or from a portion of the section by microdissection, either by scraping or by laser. The tissue is degraded by an enzyme called *proteinase K* to break open the cells and release the DNA. The sample is then purified using an automated or manual extraction kit as described below.⁹ In addition to paraffin-embedded samples, fresh or frozen tissue samples are also appropriate for DNA isolation. Quickly thawing and mincing the frozen tissue prepares the sample for DNA isolation. The minced tissue is mixed with an extraction buffer to release the DNA from the cells, and it is then purified.

There are a number of automated extraction systems as well as manual extraction kits available for DNA extraction. Most of these systems use a solid phase extraction system that takes advantage of the binding of DNA to silica under high salt conditions. Manual kits use columns that can be spun in a microcentrifuge with the eluent collected in microfuge tubes. Cells that have been lysed and protease treated are applied to a column in a high salt buffer. The column is washed to remove impurities and the DNA eluted in a low-ionic-strength buffer and collected in a microfuge tube.¹⁰ Automated extractors have reagents packaged in sets and can be programmed to extract and purify the DNA automatically. There are a variety of models to choose from, depending on the number and type of samples. Isolated DNA can be stored at -20°C . If a delay in the molecular testing is necessary, the isolated DNA sample can be stored at -80°C indefinitely.

Isolating RNA from Clinical Specimens

RNA isolation poses greater technical challenges than DNA isolation. Ubiquitous *ribonucleases* (RNases) degrade RNA. These enzymes are the body's primary defense against pathogens and are found on mammalian epidermal surfaces; therefore, they contaminate all laboratory surfaces.¹¹ Clinical laboratories that isolate RNA must be RNase free, which necessitates costly precautions and decontamination steps.¹²

The isolated total RNA includes mRNA, rRNA, and tRNA, all of which participate in protein synthesis. Depending on cell type, mRNA may comprise only 3% to 5% of the total cellular RNA; therefore, a large specimen may be needed to obtain adequate mRNA. The mRNA does not represent all the information stored in the DNA, only those genes being expressed. Consequently, mRNA provides quantitative information on the genes being expressed in a cell at the time the specimen is collected.

RNA may be purified using either liquid or solid phase procedures. The steps of RNA isolation using a liquid phase

method are (1) RNA release by cell lysis combined with RNase inhibition by homogenization or incubation in a strongly denaturing solution containing chemical agents such as urea or guanidine isothiocyanate, (2) protein and DNA removal, and (3) RNA precipitation using alcohols. In step 2, extraction is performed using acidic phenol chloroform and guanidine isothiocyanate.

These separate the DNA and protein into the organic phase, while the RNA remains in the aqueous phase. RNA resists acidic pH, whereas DNA is readily depurinated because acid cleaves the bond between the purine base and the deoxyribose sugar. Therefore, acidic phenol preferentially isolates and preserves RNA, while the genomic DNA (all the DNA) is partitioned along with contaminating proteins, lipids, and carbohydrates. Precipitating the RNA from the aqueous phase requires the addition of salt to neutralize the charge of the phosphodiester backbone and ethanol to make the nucleic acid insoluble.^{13,14} Purification of RNA using column-based methods is similar to DNA except that the RNA is suspended in a high-salt buffer that preferentially binds RNA greater than 200 nucleotides to the column to remove smaller RNAs such as tRNA and 5S RNA.

AMPLIFICATION OF NUCLEIC ACIDS BY POLYMERASE CHAIN REACTION

Polymerase Chain Reaction for Amplifying DNA

Polymerase chain reaction (PCR) is the principal technique in the clinical molecular laboratory. PCR is an enzyme-based method for amplifying a specific target sequence to allow its detection from a small amount of highly complex material.¹⁵ Sickle cell anemia results from a single β -globin nucleotide substitution (point mutation) in which an adenine replaces a thymine. Detecting this mutation from among 6 billion nucleotides in the human genome would be like finding a needle in a haystack if only a few cells were assessed. When millions of β -globin copies are produced, however, the mutation is easily detected.¹⁶ There are two categories of PCR reactions: endpoint PCR and real-time PCR. The amplification for both categories is basically the same. The major difference is in the method of detection of the PCR product. With endpoint or standard PCR, the amplification products must be detected using another technique such as gel electrophoresis, which is described later in the chapter. In real-time PCR, the amplicons are detected during the PCR cycles by using fluorescence detection. This is also described in further detail later in the chapter.

As with natural DNA replication, PCR amplification requires primers that anneal (bind) to complementary nucleotide sequences on either side of the target region. In testing for the sickle cell mutation, for example, selected primers flank (i.e., bind on either side of) the β -globin gene sequence containing the mutation. The total base pair (bp) length of the primer sequences plus the target sequence can vary, but in this example, it is 110 bp for the β -globin gene, a typical sequence length for many mutation sites (Figure 31-11).¹⁶ Besides

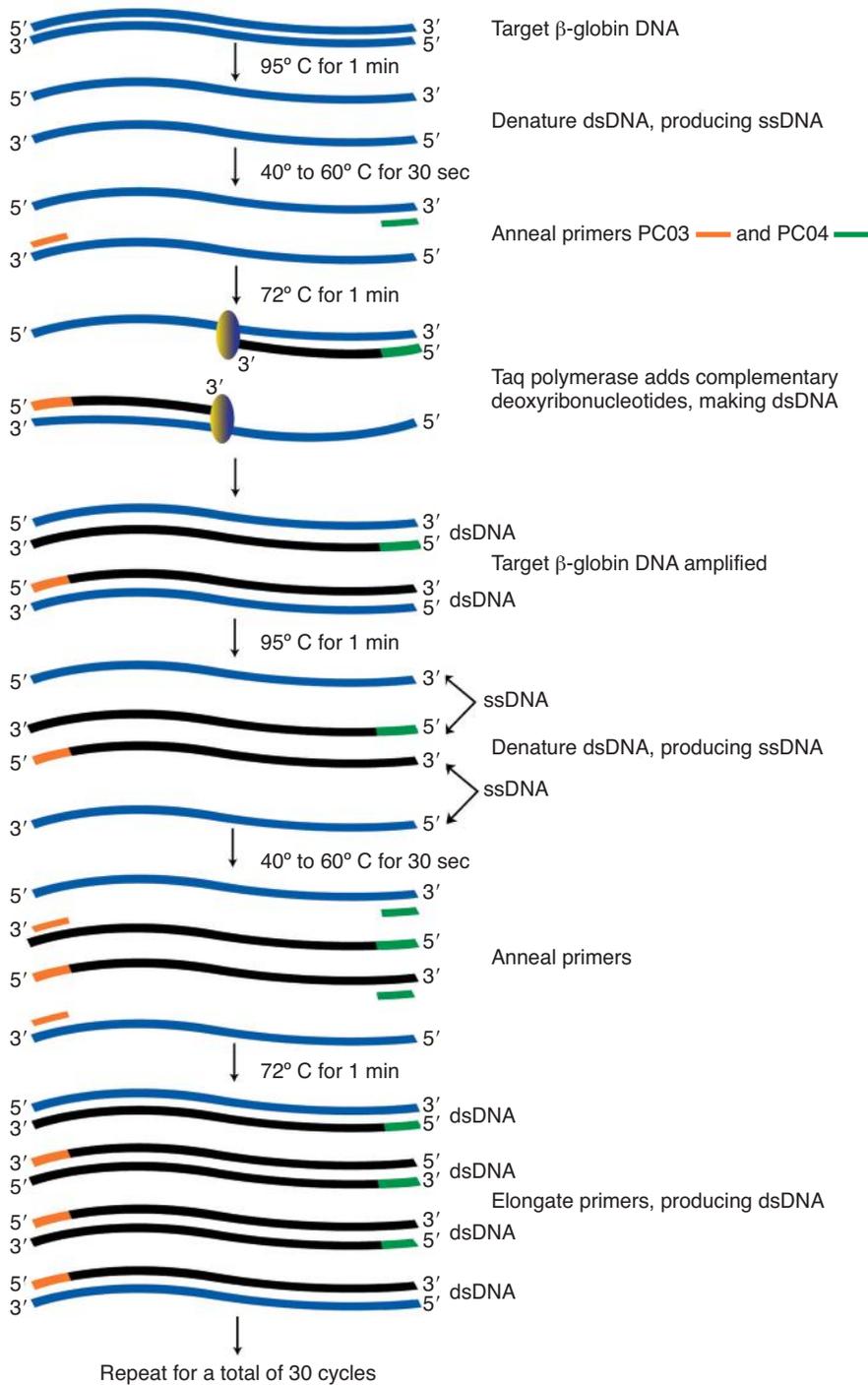


Figure 31-11 Application of PCR to target β -globin DNA. PCR amplifies the target DNA, making millions of copies of the target DNA after 30 cycles. Flanking forward and reverse primers (PC03 and PC04) are used to amplify the target β -globin DNA. One primer (PC03, orange) anneals to the 3' end of the 3'-to-5' DNA strand. The other primer (PC04, green) anneals to the 3' end of the 5'-to-3' DNA strand. These primers provide the 3'-OH end for extension in the 5' to 3' direction during the PCR reaction and set the boundaries for the size of the amplicon. *dsDNA*, double-stranded DNA; *ssDNA*, single-stranded DNA.

primers, the PCR *master mix* reagents include a heat-insensitive DNA polymerase—for example, *Taq polymerase*—isolated from the thermophilic bacterium *Thermus aquaticus* and a mixture of the four deoxyribonucleotides—deoxyadenosine triphosphate (dATP), deoxythymidine triphosphate (dTTP), deoxyguanosine triphosphate (dGTP), and deoxycytidine triphosphate (dCTP)—in a magnesium buffer.

The DNA is first *denatured* at 95° C, which separates the strands; then cooled to the primer *annealing (binding) temperature* of 40° to 60° C; and then warmed to 72° C to promote specific chain *extension*, in which nucleotides are added to the

primers by DNA polymerase. The annealing temperature is optimized for each set of primers. A *thermocycler* is used to accurately produce and monitor the rapid temperature changes.

Once the double-stranded DNA is denatured, one primer anneals to the 3' end of the 5'-to-3' strand and the other primer to the 3' end of the complementary 3'-to-5' strand. Both primers possess a free 3' hydroxyl group. The DNA polymerase recognizes this hydroxyl group, reads the template, and catalyzes formation of the phosphodiester bond joining the first complementary deoxyribonucleotide to the primer. The polymerase rapidly continues down the template strand at

1000 nucleotides per second, extending the complementary strand in the 5'-to-3' direction to eventually produce a complete daughter strand that continues to the 5' end of the template.¹⁷ This completes one PCR cycle. In the second cycle, the temperature changes are repeated, and the first-cycle product becomes the template for a daughter strand. After the second cycle, daughter strands are produced that are bounded by the primer sequences at the 5' and 3' ends, resulting in a fragment of DNA of the desired length. In 25 to 40 subsequent cycles, this DNA of specific length and sequence, called an *amplicon*, is reproduced millions of times.^{18,19}

Primer annealing accounts for PCR specificity, and primer design is crucial for achieving confidence in the test results regardless of the application. Wherever primers anneal, specifically or nonspecifically, they become starting points for extension.

Commercial kits contain primer sets that have been tested for annealing specificity, but care must be taken to use the optimal annealing temperature. Even if the primer is properly designed, it can anneal to noncomplementary regions if the annealing temperature is too low. Several online primer design programs are available from genome centers and company websites. One such program that can help determine the uniqueness and therefore specificity of the primers is the Basic Local Alignment Sequence Tool (BLAST).²⁰ These programs will also analyze pairs of primers to avoid complementarity between the primers themselves to prevent hybridization to one another, which forms undesirable *primer dimers*.

Controls are essential for the accurate interpretation of a PCR result. The three controls required for PCR are the negative, positive, and "no-DNA" or no template (NTC) controls. All three are included in each run. In addition, in most applications, a sensitivity control will be included that consists of a low positive sample at the lowest concentration detected. The negative control consists of DNA known to lack the sequence of interest; the positive control contains the target sequence. Comparison of the amplification in the patient sample to results in the negative and positive controls determines whether the target DNA sequence is present in the patient's DNA. The no-DNA control detects master mix contamination. Amplification in the no template control indicates DNA contamination, which renders the entire test result unreliable.²¹

Reverse Transcription Polymerase Chain Reaction for Amplifying RNA

Some hematology molecular tests such as those for translocations, require mRNA as the starting material. Genetically altered mRNA sequences often translate to an altered protein. The classic example is the Philadelphia chromosome (Ph'), carrying the chromosome translocation t(9;22)(q34;q11.2) (Chapter 33). This translocation is present in 95% of chronic myelogenous leukemia (CML) cases, as well as 20% of adult acute lymphoblastic leukemia (ALL) and 5% of pediatric ALL cases, and in rare instances in acute myeloid leukemia.^{22,23} Ph' results from a reciprocal translocation of the *ABL1* (Ableson) gene on chromosome 9 to the breakpoint cluster region (*BCR*) of chromosome 22, producing a *BCR-ABL1* hybrid or chimeric gene (Figure 31-12A).²⁴⁻²⁶ Transcription of *BCR-ABL1* produces

a chimeric mRNA made up of exons from both the *BCR* and *ABL1* genes. Translation generates a fusion protein, *tyrosine kinase*, that alters normal cell cycle control, which results in unrestrained cell proliferation.²⁷ RT-PCR of the chimeric mRNA is the standard method to detect this mutation. Although the mutation is present at the DNA level, the nucleotide position at which the two chromosome sections join is variable, whereas the chimeric mRNA is always the same. The DNA also includes untranslated introns, which make the chimera too long to replicate. The physiologic excision and splicing of mRNA yields a much shorter target that is more easily amplified.

In RT-PCR, the *reverse transcriptase* enzyme produces *complementary* DNA (cDNA) from mRNA present in a total RNA sample extracted from patient specimens such as blood or bone marrow (Figure 31-12). PCR subsequently amplifies the cDNA.

The first step is to transcribe the RNA into DNA using reverse transcriptase and a primer to produce an RNA-cDNA hybrid. The primer can be *oligo(dT)*, a series of thymine nucleotides complementary to the string of adenine nucleotides on the 3' end of most mRNAs, called the *polyA tail*; a set of short random primers that prime the cDNA synthesis more evenly; or a specific primer for the gene of interest. The primer anneals to the complementary sequence of the mRNA. Reverse transcriptase recognizes the hydroxyl group on the last nucleotide of the primer and reads the mRNA template strand, then adds the correct complementary deoxyribonucleotide. Reverse transcriptase continues along the mRNA template strand, joining the complementary deoxyribonucleotides to the growing cDNA strand to form the mRNA-cDNA hybrid. Subsequently, heat denaturation breaks the hydrogen bonds between the mRNA-cDNA hybrid, separating the two strands. The cDNA strand then acts as a template for replication by DNA polymerase. The cDNA synthesis can be done separately from the PCR amplification step in a two-step procedure or combined with the PCR in a single reaction. For example, with the *BCR-ABL1* translocation, the single-stranded cDNA is amplified as in DNA-based PCR using one primer specific for a target sequence in the *BCR* gene and a second primer specific for the *ABL1* gene. DNA polymerase extends the primers, forming a double-stranded cDNA of the target chimeric gene. Only the cDNA containing the translocation, and therefore both primer binding sites, will be amplified, resulting in millions of copies of the *BCR-ABL1* sequence.^{28,29}

DETECTION OF AMPLIFIED DNA

Although many molecular tests are now performed using real-time PCR, there are still circumstances where amplicons are produced using endpoint PCR and the product must be detected using downstream techniques. Amplified target DNA may be detected by gel electrophoresis using fluorescent dyes. PCR can also be combined with restriction enzyme digestion of the amplicons followed by gel electrophoresis or cleavage-based signal amplification (Invader) technology for detection (discussed later in the chapter).

Gel Electrophoresis

Nucleic acid phosphate groups confer a net negative charge to DNA fragments. Consequently, in electrophoresis, the rate at

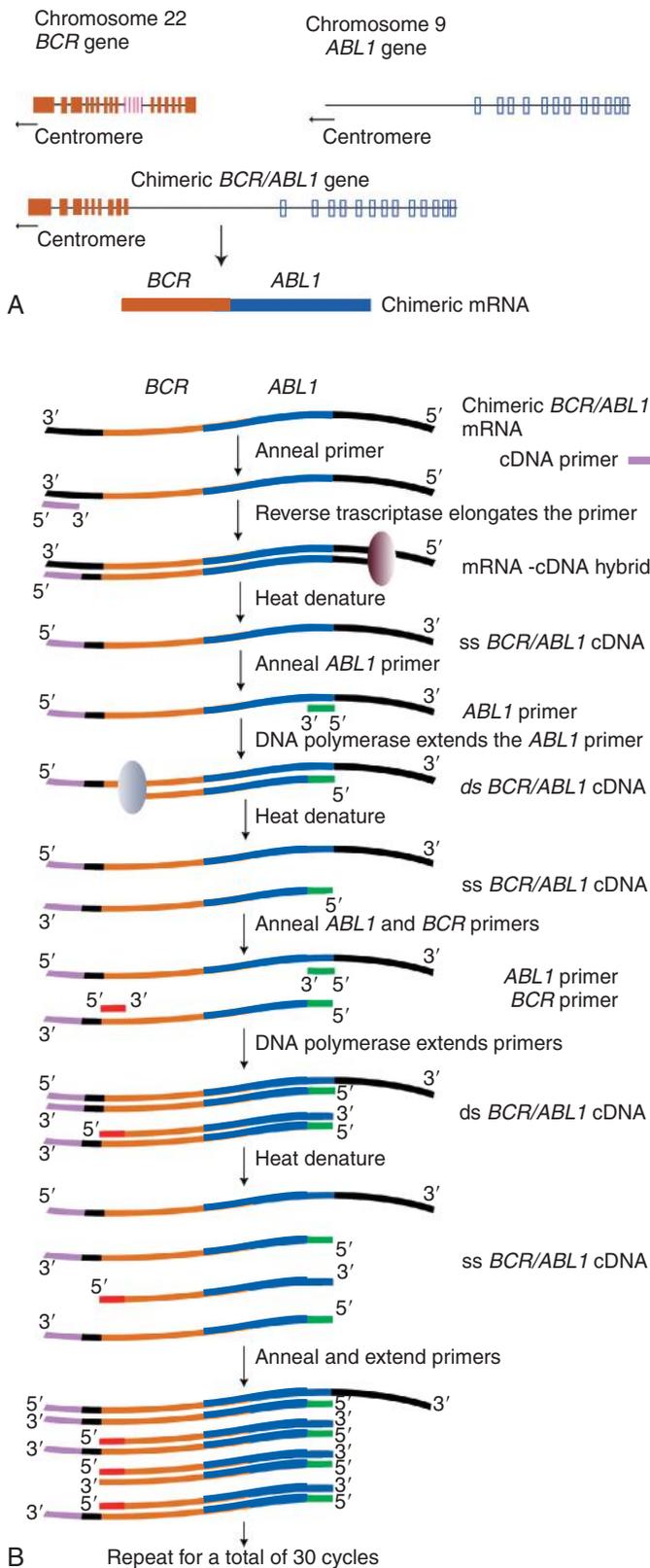


Figure 31-12 A, The *BCR* gene is present on chromosome 22, and the *ABL1* gene is located on chromosome 9. The Ph⁺ chromosome results from the translocation of the *ABL1* gene to chromosome 22, which places the *ABL1* gene next to the *BCR* gene and produces a chimeric *BCR/ABL1* gene. The transcription of the *BCR/ABL1* gene produces a chimeric messenger RNA (mRNA) consisting of a portion of the *BCR* gene and a portion of the *ABL1* gene. **B**, Reverse transcriptase polymerase chain reaction (RT-PCR) produces complementary DNA (cDNA) from messenger RNA (mRNA). This diagram shows the RT-PCR steps used to produce amplified *BCR/ABL1* cDNA. Initially, a gene-specific primer or short random primers anneal to the chimeric *BCR/ABL1* mRNA. Reverse transcriptase elongates the primer, producing an mRNA-cDNA hybrid. Heat denaturation breaks the hydrogen bonds, holding the hybrid molecule together, releasing the single-stranded (ss) *BCR/ABL1* cDNA. Next, a primer specific for the *ABL1* gene is annealed to the cDNA. DNA polymerase elongates the primer, producing the double-stranded (ds) *BCR/ABL1* cDNA. The cDNA becomes single stranded by heat denaturation. Then the *ABL1* primer as well as a primer specific for the *BCR* gene anneal to the ss cDNA. DNA polymerase elongates the primers, producing ds *BCR/ABL1* cDNA. The cycle is repeated 20 to 40 times, producing millions of copies of the ds *BCR/ABL1* cDNA.

which DNA fragments (amplicons) migrate through gels is proportional to their mass only and, unlike proteins, not their relative charge. DNA fragment mass is a function of the length in base pairs (bp) or kilobase pairs (kb, 1000 × bp). Fragments are sieved through an agarose or polyacrylamide gel matrix by passing a current through the gel as it is bathed in a buffered conducting salt solution. Electrophoresis gel pore diameter is a function of gel concentration. The pores of an agarose gel are larger than the pores of a polyacrylamide gel. When larger fragments (500 bp to 50 kb) are to be separated, an agarose gel is most effective. For smaller DNA fragments (5 to 1000 bp), a polyacrylamide gel is used.³⁰

In slab gel electrophoresis, PCR products (amplicons) of patients and controls, and a mass marker or ladder are pipetted into the sample wells in the gel slab near the negative electrode (cathode). An electrical current moves the negatively charged fragments toward the positive electrode (anode). Smaller fragments move faster and migrate farther than larger fragments. The ladder, composed of fragments of known masses (sizes), measured in base pairs or kilobase pairs, runs alongside the patient and control lanes and is used to determine the mass (size) of any DNA fragments in the patient and control samples (Figure 31-13). Fluorescent dyes such as ethidium bromide and Gel Red[®] (which intercalate between the base pairs of the DNA helix) or SYBR green[®] (which binds to the minor groove of the DNA helix) are employed to visualize the DNA fragments of the patient, controls, and size markers in the gel. The newer dyes (e.g., Gel Red and SYBR green) have largely replaced ethidium bromide because they are much less toxic. Gels are soaked in a solution of diluted dye and then exposed to UV light, which causes the nucleic acid to appear as fluorescent bands. The mass of the bands in the patient and control lanes is determined by comparing the distance they migrated in the gel with the distance migrated by the bands of the size markers. Gel electrophoresis is appropriate

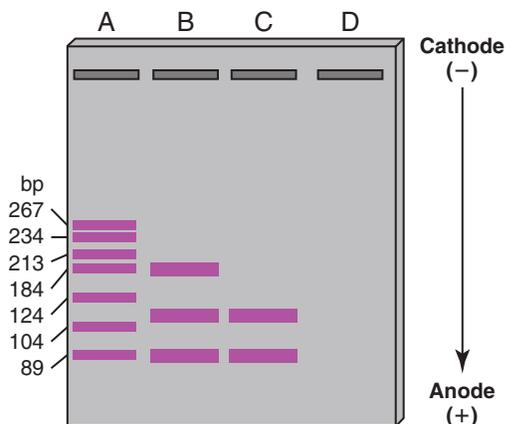


Figure 31-13 Electrophoresis pattern of a DNA sample on a slab gel. **A**, Molecular size marker (ladder); **B**, positive control; **C**, negative control; **D**, no-DNA control. DNA samples are placed in wells at the cathode (negative pole) and migrate to the anode (positive pole) due to the negative charge of the DNA molecules. By comparing the bands present in the gel with the molecular size markers, the mass of each band, measured in base pairs, is determined. For example, in the positive control sample, the three bands are 184, 110, and 89 bp. Positive, negative, and no-DNA controls must be used when performing gel electrophoresis. The positive control contains the target DNA sequence, and the negative control lacks this sequence. The no-DNA control sample lacks DNA. No banding should be present in the no-DNA control. If bands are present, contamination of samples occurred during the testing process.

when the goal is qualitative—that is, to determine the presence or absence of the target DNA.

Another method of fractionating DNA fragments by mass (size) is capillary gel electrophoresis. In this type of electrophoresis, long fused silica capillaries filled with derivatized acrylamide polymer are used for separation of single-stranded negatively charged DNA fragments on the basis of size or number of base pairs. The sample is applied to the capillary using electrokinetic injection, and the DNA fragments are separated using a high voltage as they migrate through the capillary from the negative electrode to the positive electrode. Smaller DNA fragments

move faster through the polymer in the capillary compared to larger fragments. Detection of the separated fragments occurs by incorporating a fluorescent label into the PCR-amplified DNA. Before reaching the positive electrode the fluorescently labeled DNA fragments cross the path of a laser beam and detector. When the laser beam hits a fluorescent DNA fragment, light is emitted at a specific wavelength. The light emission is read by the detector, and the signal produces a peak on an electropherogram (Figure 31-14).

Capillary electrophoresis offers a number of advantages over traditional gel electrophoresis. The injection, separation, and detection of the fragments are automated. The separation can be quite rapid with excellent resolution. The time of fragment elution and the peak height information are stored for easy retrieval. Size ladders can be labeled with different fluorescent dyes and run in the same capillary as the sample providing more accurate sizing.³¹ This method of separation is used in a number of applications including B and T cell clonality testing, bone marrow engraftment analysis, and screening for the internal tandem duplication mutation in the *FLT3* gene in acute myeloid leukemia (AML) (Chapter 35).³²

Restriction Endonuclease Methods

One method to determine whether an amplified target DNA fragment contains a mutation of interest uses enzymes called *restriction endonucleases* (also known as *restriction enzymes*). These enzymes are produced naturally in bacteria and are so named because they restrict foreign (phage) DNA from entering and destroying the bacterium. Each restriction enzyme recognizes a specific nucleotide sequence and cuts both strands of the target DNA at the sequence, producing *restriction fragments*. *Recognition sequences* can be 4 to 15 nucleotides long. There are hundreds of commercially available restriction endonucleases, which allow recognition of many sequences. The number of restriction fragments produced depends on the number of restriction sites present in the amplified target.^{33,34} Enzyme action at one restriction site produces two restriction

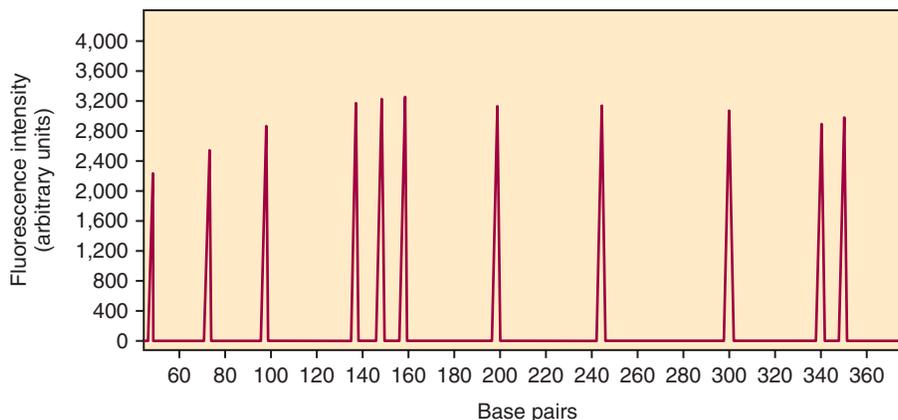


Figure 31-14 An electropherogram of capillary gel electrophoresis showing the separation of fluorescently labeled DNA fragments by size (number of base pairs). Fragments migrate through the matrix in the capillary from the negative to the positive pole and emerge from the capillary in size order (smaller fragments first). Before reaching the positive electrode, the fluorescently labeled DNA fragments are detected by passing one at a time between a laser beam and the detector. Each fragment is represented by a peak on the electropherogram.

fragments, action at two restriction sites produce three restriction fragments, and so on.

A restriction enzyme detects even a single base substitution if the mutation alters the recognition sequence and prevents digestion at the site or creates a new site resulting in an additional fragment. A *restriction fragment length polymorphism* (RFLP) is a mutation or polymorphism-induced change in the recognition site of the restriction enzyme that alters the length (number of base pairs) of the restriction fragment. A mutation in the coagulation factor V gene (*F5* Leiden mutation) is an excellent example of RFLP. Individuals possessing this mutation have an increased risk of venous thrombosis. This mutation results from the replacement of guanine with adenine at nucleotide position 1691 (G1691A) of the *F5* gene.^{35,36} The mutation alters a restriction site normally detected and cut by the restriction enzyme *Mnl* I. The wild-type (normal) *F5* amplicon is 223 bp long with two *Mnl* I-specific sites. After PCR amplification and incubation with *Mnl* I, the

wild-type amplicon is cut into three restriction fragments, separable using slab gel or capillary electrophoresis. The fragments are 37, 82, and 104 bp long. The mutant gene generates only two fragments with lengths of 82 and 141 bp. A sample from an individual homozygous for the wild-type gene generates the three expected fragments 37, 82, and 104 bp. A sample from an individual homozygous for the *F5* Leiden mutation possesses two copies of the mutated *F5* gene and generates only two bands: 82 and 141 bp. A sample from a heterozygous individual possesses one normal and one mutated *F5* gene and produces four bands of lengths 37, 82, 104, and 141 bp (Figure 31-15 and Figure 31-1).

Nucleic Acid Hybridization and Southern Blotting

Once used in a number of molecular tests, including B and T cell clonality assays, as well as the detection of chromosome translocations, Southern blots are now largely used for samples

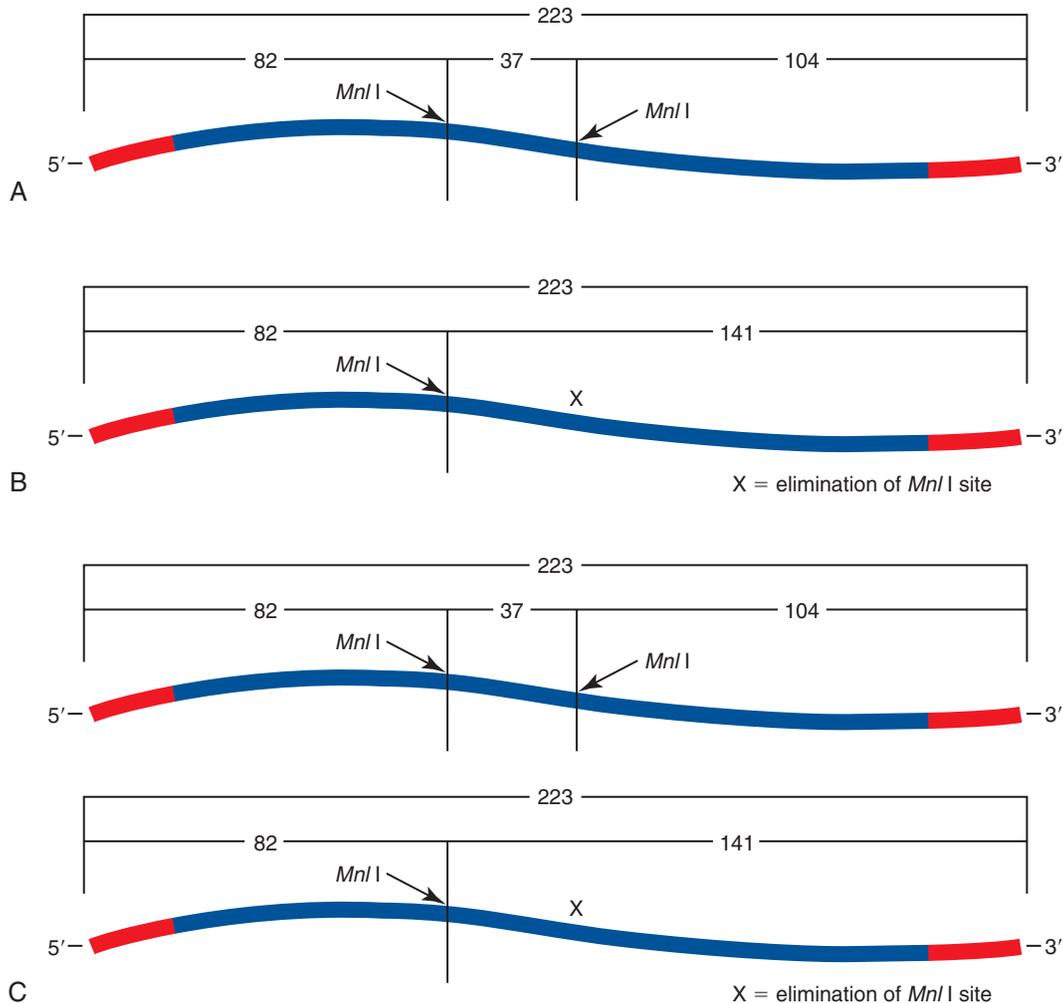


Figure 31-15 Diagram of the amplified target sequence of the coagulation factor V (*F5*) gene (223 bp) in a PCR-Restriction Fragment Length Polymorphism (RFLP) method to detect the *F5* Leiden mutation. **A**, The amplified target sequence for the normal (wild-type) *F5* gene contains two restriction sites for *Mnl* I, so three restriction fragments of 37, 82, and 104 bp are produced. **B**, In the *F5* Leiden mutation, the substitution of an A for a G in position 1691 of the *F5* gene eliminates one of the restriction sites for *Mnl* I. Thus the mutated *F5* Leiden gene possesses only one restriction site for *Mnl* I. In individuals homozygous for the mutation, only two restriction fragments of 82 and 141 bp are produced. **C**, An individual who is heterozygous for the *F5* Leiden mutation has a normal and a mutated *F5* gene. *Mnl* I produces four restriction fragments of 37, 82, 104, and 141 bp.

that do not provide a result using standard PCR methods or for research applications. Southern blots can only be performed on high-quality genomic DNA or PCR amplicons. Briefly, DNA is digested with a restriction enzyme, size fractionated using agarose gel electrophoresis, denatured to become single stranded, and then finally transferred to a solid support—typically a nylon or nitrocellulose membrane and then detected with a labeled probe.

In the classic Southern blot procedure, detection of the band containing the sequence of interest requires a radioactive or enzyme (horseradish peroxidase or alkaline phosphatase)–conjugated, single-stranded probe complementary to the target sequence. The probe hybridizes to the target DNA, unhybridized probe is washed off, and the hybridized bands are visualized, depending on the labeling system chosen. Most probes today are detected using a chemiluminescence detection by autoradiography (Figure 31-16).³⁷⁻⁴¹

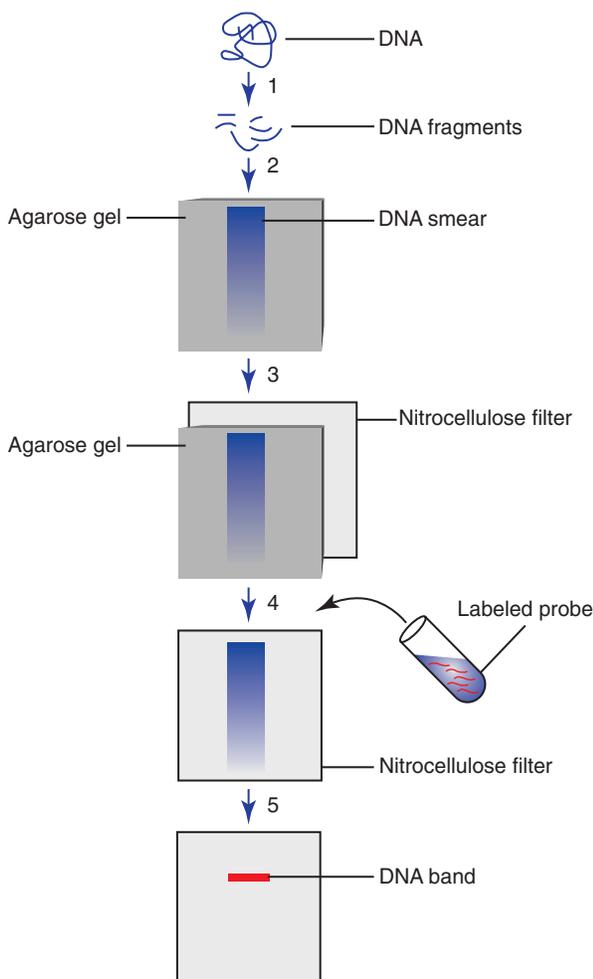


Figure 31-16 Southern blot steps. 1, DNA is cut with the restriction endonuclease *EcoRI*, which produces many restriction fragments. 2, DNA fragments are separated on an agarose gel. 3, DNA fragments are transferred to a nitrocellulose filter. 4, A labeled probe is hybridized to the DNA fragments on the filter. 5, Autoradiography is used to visualize the hybridized DNA probe, the detection of which indicates the presence of the given DNA sequence in the sample.

Cleavage-Based Signal Amplification

Cleavage-based amplification is an isothermal signal amplification method marketed as Invader[®] (Hologic, Inc., Bedford, MA). In the primary reaction, the 3' end of a test probe and an Invader oligo probe anneal to complementary sequences on the target DNA template forming a specific substrate site recognized by a Cleavase enzyme (Figure 31-17). The 5' end of the test probe (5' flap) does not anneal to the target. The Cleavase enzyme cuts and releases the 5' flap of the test probe. In a coupled secondary reaction, the 5' flap of the test probe anneals to a complementary signal probe that has a FRET (fluorescence resonance energy transfer) reporter. The signal probe and reporter (called a FRET cassette) is specific for the 5' flap of the test probe. The FRET reporter is a fluorescent dye bound to the signal probe in close proximity to a quencher; the quencher prevents the reporter dye from emitting a fluorescent signal. The combination of the 5' flap of the test probe and the FRET cassette forms another specific substrate site for the Cleavase enzyme. Cleavage of the 5' end of the FRET cassette results in separation of the fluorescent reporter from the quencher and production of a fluorescent signal. Repeated binding and cleavage result in the signal amplification. Two reactions are done simultaneously using different fluorescent molecules for detection of either the wild-type or mutant sequence. Because the mutant sequence is different from the wild-type sequence, the wild-type test probe will not anneal to the mutant target, and no fluorescent signal will occur. This technique can be used to detect single base pair changes, small insertions, and deletions. It is also FDA-approved for the detection of mutations associated with thrombophilia, including the factor V (*F5*) Leiden mutation, the prothrombin G20210A mutation in the *F2* gene, and methylenetetrahydrofolate reductase (*MTHFR*) gene mutations.⁴²

DNA Sequencing

The ability to read the sequence of the nucleic acid has been just as important as PCR in the development of molecular biology. A combination of these two important techniques (cycle sequencing) has made DNA sequencing an integral part of molecular diagnostics. In cycle sequencing, the order of the nucleotide bases is determined after amplification.⁴³ Cycle sequencing is applied in molecular testing to assess amplified sequences for insertions, deletions, or point mutations, such as the *FLT3* internal tandem duplication (ITD) or point mutations in the *KIT* gene that occur in AML (Chapter 35).⁴⁴⁻⁴⁶

Cycle sequencing is based on dideoxynucleotide terminator sequencing.⁴⁷ The addition of nucleotides to a growing DNA polymer requires a 3' hydroxyl group on the last added nucleotide and a triphosphate group on the 5' end of the next nucleotide to be added (Figure 31-5). If a nucleotide lacks the 3' hydroxyl group, it can be incorporated into the newly synthesized strand of the DNA but cannot be extended, so the fragment terminates at the “defective” base. If low concentrations of the terminators, dideoxyadenosine triphosphate, dideoxycytosine triphosphate, dideoxyguanine triphosphate, and dideoxythymine triphosphate, are included in the single primer PCR master mix used for sequencing, over a number of cycles

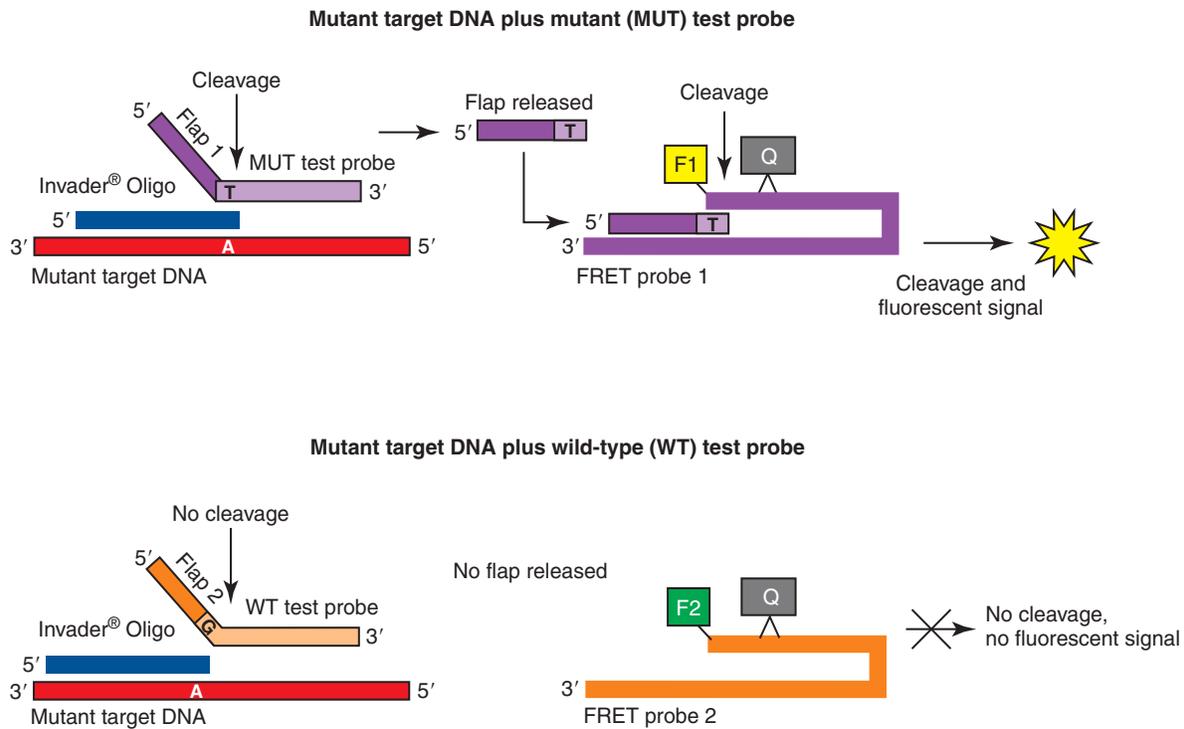


Figure 31-17 Cleavage-based signal amplification. The Cleavage-based DNA Signal Amplification Assays use a Cleavase[®] enzyme to recognize and cleave specific structures formed by the addition of two oligonucleotide probes to a nucleic acid target. Two oligonucleotide probes (a test probe and an Invader[®] oligo probe) hybridize in tandem to the target DNA to form an overlapping structure. The 5' end of the test probe includes a 5' flap that does not hybridize to the target DNA. The 3' nucleotide of the bound Invader[®] oligo overlaps the test probe. The Cleavase[®] enzyme recognizes this overlapping structure and cleaves off the unpaired 5' flap of the test probe, releasing it as a target-specific product. In the secondary reaction, each released 5' flap anneals to a fluorescence resonance energy transfer (FRET[™]) probe to create another overlapping structure that is recognized and cleaved by the Cleavase[®] enzyme. When the FRET[™] probe is cleaved, the fluorophore (F) and quencher (Q) on the FRET[™] probe are separated, generating detectable fluorescence signal. The initial and secondary reactions run concurrently in the same well. Two different fluorescent labels are used, one to identify the presence of the wild-type allele and one to identify the presence of the mutant allele.

a series of DNA fragments is produced that terminate at each successive base with each fragment differing in length by one nucleotide. This is called a *ladder* or *nested series* of fragments.

In the dye terminator method each of the four dideoxynucleotides in the PCR reaction is labeled with a different fluorescent dye so that each DNA fragment terminates in a labeled dideoxynucleotide corresponding to the sequence of the target DNA (Figure 31-18, A). The specific fluorescent color of the DNA fragment identifies the terminal nucleotide. Alternatively, in the dye primer method, the primers are labeled with four different fluorescent dyes (corresponding to each nucleotide), and in separate tubes, each labeled primer is subjected to PCR with unlabeled dideoxynucleotides (Figure 31-18, B). As in the dye terminator method, the specific fluorescent color of the DNA fragments corresponds to the terminal nucleotide.

The fluorescently labeled fragments are subjected to capillary electrophoresis (described earlier in the chapter). The DNA fragments migrate through the capillary and separate based on their size. Near the end of the capillary, the fragments pass one by one through the beam of a laser in an order based on their length (with the shortest fragments emerging first). A detector reads the specific fluorescent color of each fragment and displays the signal as a peak on an electropherogram; this allows the sequence to be read (Figure 31-18, C).

In order to unambiguously read the nucleotide at each position, the PCR reaction for cycle sequencing contains only a single primer that produces *single-sided* PCR. Two separate reactions are typically carried out, one using the forward primer and a second using the reverse primer. This produces complementary sequences from both strands. After the cycle sequencing reactions, the nested products are purified and denatured before loading on the capillary sequencer. The injection, separation, and detection are automated, but the operator can set the parameters such as amount of sample injected, length of capillaries, and the type of polymer. Capillary DNA sequencing instruments are equipped with base calling software that will read the base sequence of the DNA fragment sequenced. Software packages will also identify alterations in the sequence such as single nucleotide polymorphisms (SNPs), point mutations, and insertions or deletions based on comparison to a specific reference sequence.

Pyrosequencing is another sequencing method that is useful for the determination of point mutations and short sequence analysis. This method uses a “sequencing by synthesis” principle and the detection of pyrophosphate release upon nucleotide incorporation. Nucleotides are added sequentially to a single-stranded template, and when the complementary base is added, it is incorporated, resulting in pyrophosphate release. The pyrophosphate

fragments multiple times and use bioinformatics to reassemble the sequence. The process of NGS can be divided into several steps, including template preparation, sequencing and detection, and finally data analysis and assembly. Currently available commercial systems use a variety of methods. One commonly used method involves the immobilization of molecules on a solid phase followed by amplification to produce clonally amplified clusters. Sequencing by synthesis reactions are carried out using cyclic reversible terminators in four colors and fluorescent detection by lasers following each base addition. A second commonly used method also amplifies the sequencing template but uses emulsion PCR to accomplish it. The sequencing technology takes advantage of the hydrogen ion released when a base is added and uses semiconductor technology to translate that into a nucleotide sequence by the sequential addition of bases and the measurement of the voltage produced when the correct nucleotide base is added. Both methods use proprietary software and alignment to a reference sequence to produce the final template sequence. There are also numerous programs available as open source or from commercial vendors for analysis. Current applications for NGS have been mainly limited to the sequencing of panels of genes associated with a particular disease. This makes the bioinformatics analysis more manageable and limits the number of variants of unknown significance (VUS) that are identified.⁴⁹⁻⁵¹

REAL-TIME POLYMERASE CHAIN REACTION

In contrast to *end-point* PCR, *real-time* PCR measures the change in nucleic acid amplification as replication progresses using fluorescent marker dyes. There are several commercially available instruments that vary in their capacity, sample volume, and optics.⁵² There are a variety of choices in the optics for fluorescent detection. A tungsten lamp is commonly used for excitation, and different filters are used to select the excitation and emission wavelength. Light emitting diodes (LEDs) or lasers for excitation can also be coupled with emission detection, depending on the instrument. Real-time PCR can be used in quantitative or qualitative assays. The time interval, expressed as the number of replication cycles, required to reach a selected fluorescence threshold is proportional to the *copy number* of target molecules in the original sample.⁵³ The PCR cycle at which amplification crosses the threshold is denoted as the *Ct* for *threshold value* or the *Cp* for *crossing point value*. Importantly these values are calculated from the exponential portion of the amplification curve. The *Ct* value is inversely related to the amount of target so that the more starting DNA or cDNA that is present in the reaction, the lower the number of PCR cycles that are required to reach the threshold and exponential phase of the reaction (Figure 31-19).

Real-time PCR requires the use of fluorescent detection, and there are several different options available. The simplest and the most straightforward option is to add a fluorescent dye, such as SYBR® green, to the PCR reaction. These dyes bind to double-stranded DNA so that the fluorescence increases in proportion to the number of copies of the PCR product. The disadvantage of this approach is that these dyes do not differentiate between

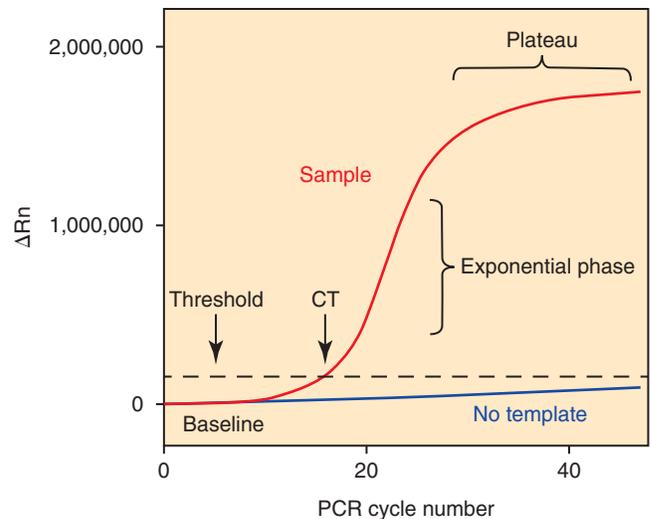


Figure 31-19 Real-time PCR amplification curve illustrating the important features of the curve, including the threshold *Ct* value and the exponential phase of the curve.

specific and nonspecific PCR amplicons, so the PCR reaction must be free of mispriming and primer dimers (primers that partially anneal to one another and are extended by the polymerase, forming very short amplicons). A more specific method of detection adds a probe in addition to the forward and reverse PCR primers, which also binds to the amplicon, providing additional specificity. There are several methods commonly employed, including hybridization probes, Taqman probes, and molecular beacon and Scorpion probes. Hybridization probes utilize two oligonucleotide probes that bind to the amplicon adjacent (within one to five bases) to one another. One of the oligos has a 3' donor fluorophore and the other a 5' acceptor. The 3' fluorophore is excited, and the energy is transferred to the acceptor, which then fluoresces at a detectable wavelength. This is called fluorescence resonance energy transfer, or FRET.⁵⁴ Hybridization probe technology in combination with melting curve analysis is used in some commercial thrombophilia assays.

Taqman probes consist of a single oligonucleotide that anneals between the forward and reverse primers. This probe contains a fluorophore on the 5' end and a quencher on the 3' end. This method takes advantage of the 5'-to-3' exonuclease activity of DNA polymerase. As the amplicon is synthesized by DNA polymerase, the probe is degraded. This separates the fluorophore from the quencher and results in fluorescence. As the number of amplicons increases, there is more target to anneal to the probe and greater fluorescence as the probes are degraded.⁵⁵ Molecular beacons and Scorpion probes use a hairpin structure to juxtapose the reporter and quencher. When the probe binds to the target, the hairpin unfolds and separates the fluorophore from the quencher, and fluorescence is detected.⁵⁶

Qualitative Real-Time Polymerase Chain Reaction

Taqman assays are used to detect point mutations such as the common point mutations in hereditary hemochromatosis (Chapter 20). Two Taqman probes are synthesized with a

different fluorescent label, complementary to either the wild-type or mutant sequence. If the sequence is complementary to the target, the probe will be degraded as described above, and fluorescence will be produced. If the sequence contains a mismatch, the probe will be displaced and the fluorescence will remain quenched. Real-time PCR can also be combined with sequence-specific primer PCR (SSP-PCR) to detect point mutations or SNPs. This method takes advantage of the fact that the 3' end of a primer in PCR must match the template sequence exactly to be extended by the polymerase. This is in contrast to the 5' end of the primer, which can have additional nucleotides added. By using primers complementary to either the wild-type or mutant nucleotide, the presence or absence of a mutation can be determined by the reaction that produces the PCR product.⁵⁷ There are several modifications of this technique. One widely used technique, called SNaPshot®, uses dideoxynucleotides, each labeled with a different fluorophore and primers of different size in a multiplex reaction to detect several different nucleotide changes simultaneously. The different-sized PCR products are then identified by size fractionation using capillary gel electrophoresis (Figure 31-20).⁵⁸ There are other variations on this technique as well that are not discussed here. It is clear, however, that there are an increasing number of applications of real-time PCR in hematology, and multiple different techniques can be used to detect the same mutation. Applications of these techniques include the detection of resistance mutations in viruses or bacteria, as well as somatic mutations in cancer cells and germline mutations in genetic diseases.⁵⁹

Quantitative Real-Time Polymerase Chain Reaction

Real-time quantitative PCR can be done in two ways: relative quantitation, which normalizes to a reference gene used when measuring gene expression, or absolute quantitation, where a standard curve of known copy number of diluted standards is run along with the patient samples. Once the relationship between the copy number of the standards and the Ct value is determined using the standard curve, the copy number of the patient sample can be determined from the crossing point value. This is used to monitor residual disease in chronic myelogenous leukemia by quantifying the amount of the BCR-ABL1 transcript (Chapter 33) as well as viral loads in infectious disease (Figure 31-21).⁶⁰⁻⁶⁶

High Resolution Melting Curve (HRM) analysis is a real-time PCR method that uses the quantitative analysis of the melting curve to detect sequence differences in PCR amplicons. Melting curves are often run in conjunction with real-time PCRs to confirm specificity of the product by heating the amplicon in increasing intervals from 65° C to 95° C and measuring the fluorescence. When the double-stranded DNA melts, the fluorescence will sharply decrease. High-resolution melt curves use narrower temperature increments and a saturating fluorescent dye to determine the melting curve. This allows the determination of sequence differences in PCR amplicons. This method requires a thermocycler with good temperature stability and a software package for HRM analysis. The advantage of HRM is that it will detect any sequence difference in an amplicon and the exact mutation does not have to be known in advance.⁶⁷

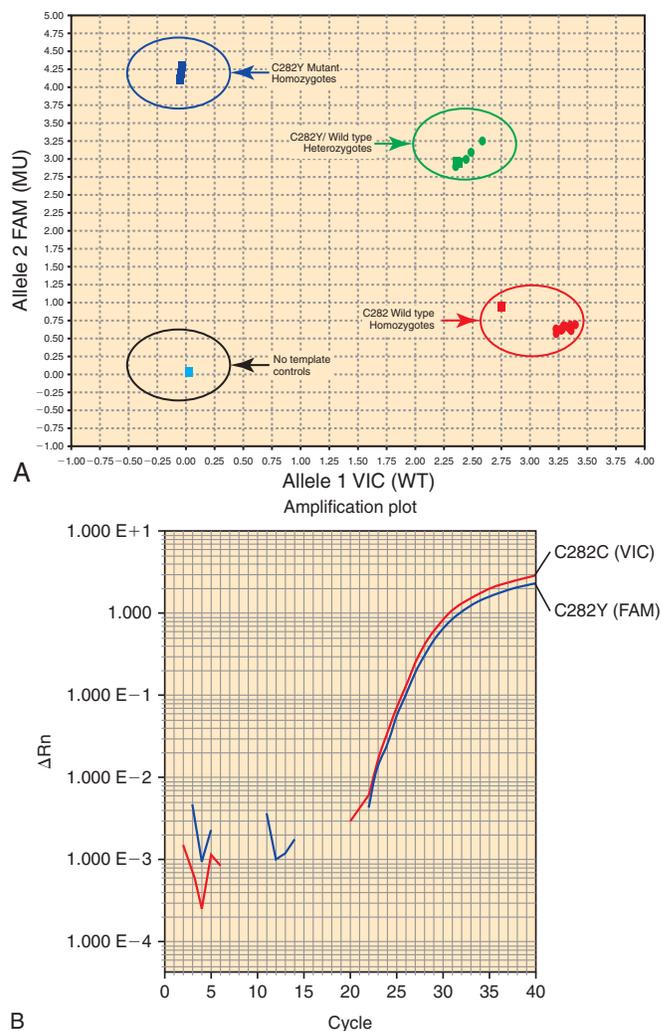


Figure 31-20 Qualitative real-time PCR for hereditary hemochromatosis, *HFE* C282 gene mutation detection. The mutation replaces the amino acid cysteine (C) with tyrosine (Y) in position 282 of the *HFE* protein. The method uses two Taqman amplification probes, one for the wild-type (normal) and one for the mutant allele, each labeled with a different fluorescent reporter. **A**, Taqman allelic discrimination plot demonstrating the three genotypic populations for the *HFE* gene and no template controls. The scatter plots are derived from the total fluorescence of the amplification curve for both fluorescent probes. The genotype can be determined from the position on the scatter plot. **B**, Real-time PCR amplification curves of a heterozygous C282C/Y mutant patient showing fluorescence with the two Taqman probes: VIC for the wild-type and FAM for the mutant allele.

Minimal Residual Disease in Leukemia

Real-time quantitative PCR provides the opportunity to follow disease burden and to measure *minimal residual disease* (MRD) in leukemia, a key indicator of treatment efficacy, clinical remission, and prognosis.^{61,62} Currently, chemotherapy, radiation therapy, and hematopoietic stem cell transplantation reduce leukemic cells to levels undetectable first by visual examination of a bone marrow smear or peripheral blood film and later by flow cytometry assay.⁶⁸ The persistence of disease after treatment that is only detected by molecular assays is called *minimal residual disease*. Real-time quantitative PCR identifies the specific nucleic acid sequence in residual leukemic cells and helps guide the types and

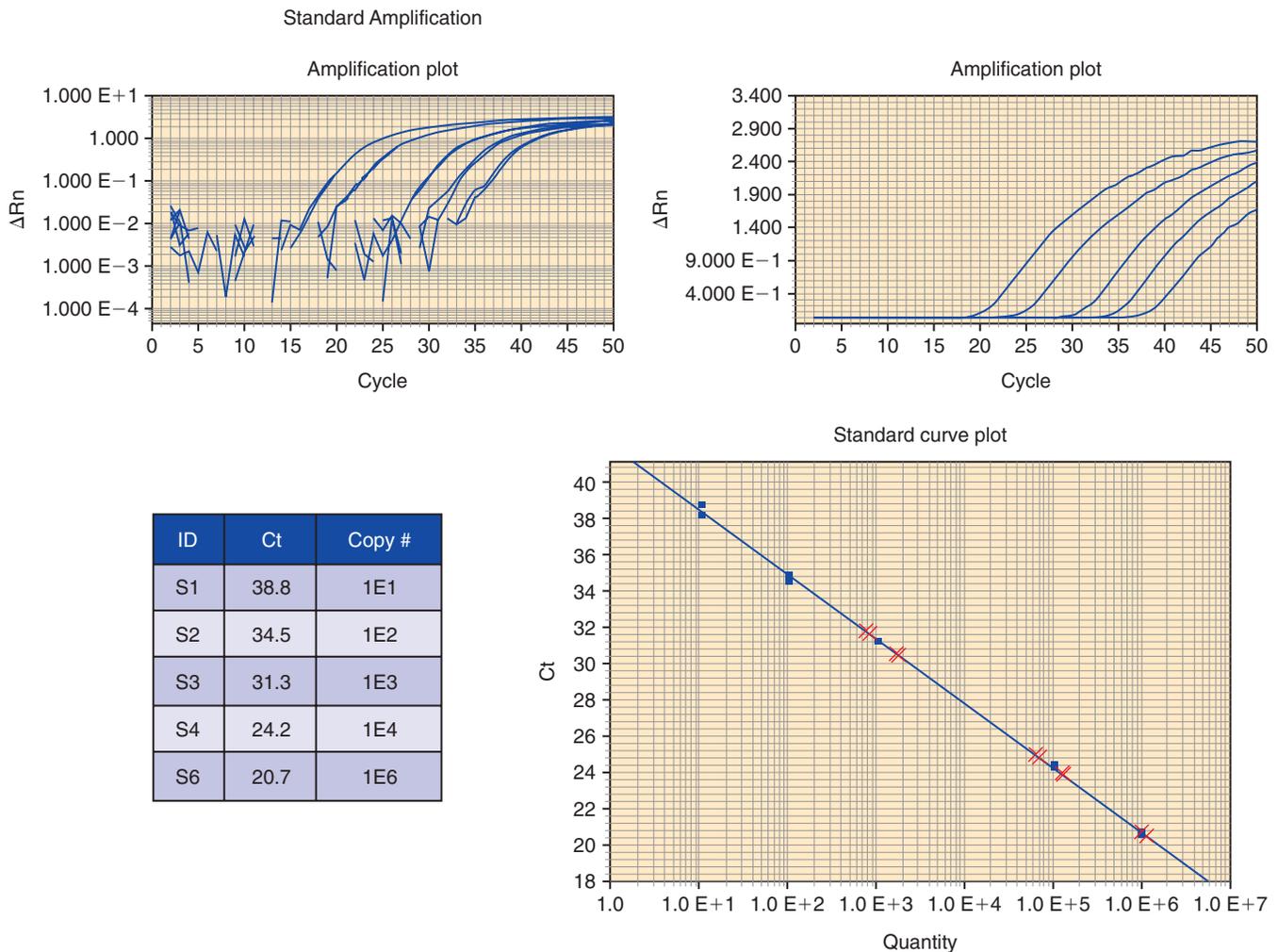


Figure 31-21 Quantitative real-time PCR for BCR-ABL1 transcript showing the standard curve with standards containing known copy numbers of the *BCR-ABL1* cDNA. Plotting the crossing point threshold value (CT) on the y -axis versus the log of the copy number on the x -axis generates a standard curve.

intensity of therapy with the goal of “molecular” remission. Real-time reverse transcriptase quantitative PCR to assess the fusion transcript levels for CML is regarded as the “gold standard” for the detection and quantification of minimal residual disease. Subsequent to remission, periodic real-time quantitative PCR assays are used to detect early relapse and drug resistance, enabling the hematologist to initiate appropriate follow-up therapy.⁶⁹

Real-time quantitative PCR may detect a few malignant cells within a population of a million cells, providing unparalleled sensitivity. Current assays to assess MRD can detect one leukemic cell among 10^5 to 10^6 normal cells. Current applications include detection of BCR-ABL1 transcripts in CML and some acute leukemias (Figure 31-22); *JAK2* (“just another kinase” or Janus kinase) mutations in the myeloproliferative neoplasms, polycythemia vera, and essential thrombocythemia (Chapter 33); the *t(15;17)* (q22;q21) or PML-RARA fusion transcript in *acute promyelocytic leukemia* (Chapter 35); and *gene rearrangements* in mature lymphoid neoplasms (Chapter 36).⁷⁰

A major issue with quantitative assays has been the lack of reproducibility between different laboratories due to the specimen type and quality, the choice of housekeeping gene for normalization, and the specific assay used. Recently an international

standard for BCR-ABL1 has been developed and made available by the World Health Organization (WHO). This standard will serve as a universal standard and allow for interlaboratory comparison.⁷¹

Mutation Enrichment Strategies

In order to detect low levels of disease or emerging resistance, it is helpful to be able to enrich for the presence of the mutation. There are currently several methods to accomplish this, all of which seek to selectively amplify the mutant sequence in the presence of an excess of wild-type sequence. Peptide-nucleic acid (PNA) and locked nucleic acid (LNA) both contain normal nucleotide bases for hybridization but different backbones from the phosphodiester backbone of DNA and RNA.⁷² This gives these probes the ability to hybridize more tightly when used as probes in PCR reactions. When the probes span and match the wild-type sequence, they can inhibit the amplification of the wild-type allele, thus enriching for the mutant allele.⁷³

COLD-PCR (co-amplification at lower denaturation temperature-PCR) is another mutation enrichment technique based on PCR amplification. COLD-PCR is based on the principle that DNA containing a mismatch will melt at a

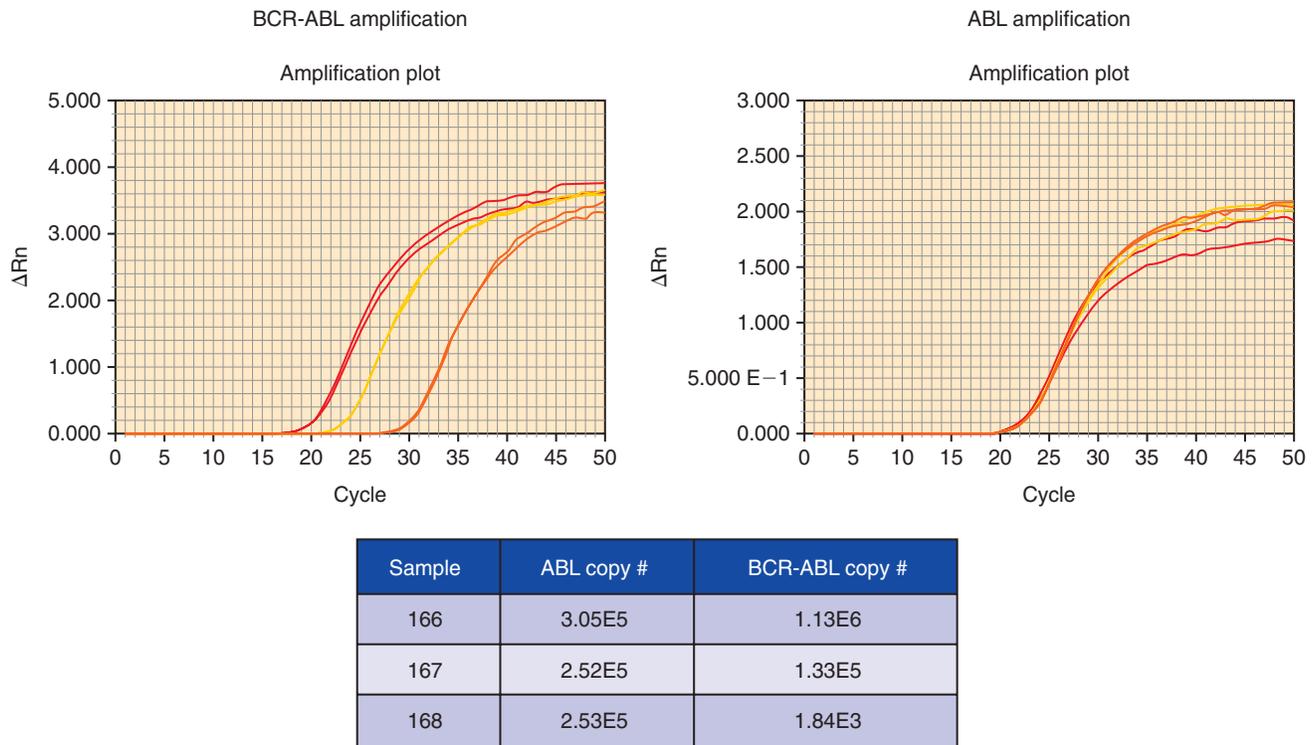


Figure 31-22 Quantitative real-time PCR for BCR-ABL1 transcript. Using the standard curve, the copy number of BCR-ABL1 transcripts in unknown samples can be determined. Two amplification graphs are shown. The graph on the left shows the amplification of the BCR-ABL1 transcript, and the graph on the right shows the amplification of the ABL1 transcript as a control for sample quality.

slightly lower temperature than completely matched sequences. Designing the PCR cycle temperatures to maximize that difference results in a preferential amplification of the mutant sequence in a mixed sample of mutant and wild-type DNA, even when the mutant is in very low concentrations. This is accomplished by carrying out the denaturation step at the temperature that will have mutant-wild-type heteroduplexes in a single-stranded state, while wild-type homoduplexes will not yet have denatured.⁷⁴ All of these methods, although useful to enrich for mutant alleles, are technically demanding and therefore are not yet in widespread usage in molecular laboratories.

CHROMOSOME MICROARRAYS

Chromosomal microarray analysis is a methodology used to measure gains and losses of genomic DNA. The advantage of microarrays compared to karyotyping is that it is a higher-resolution method and will detect genetic changes that cannot be observed by karyotyping (Chapter 30). In addition, chromosome microarrays have the advantage of also detecting aneuploidy and large chromosomal duplications and insertions.

There are two different types of chromosome microarrays: comparative genomic hybridization (aCGH) and single nucleotide polymorphism array (SNP-A) karyotyping. Both types of arrays can identify variation in copy number. Due to differences in methodology, however, they detect different types of variants (Figure 31-23).

In an array-based assay, the specimen DNA is isolated, denatured, and hybridized to a chip or array containing thousands

of probes with known sequences. For comparative genome hybridization, the patient and a control DNA are labeled with different fluorescent dyes, and after hybridization, the relative intensity of the two fluorescent signals is used to determine if there are any genomic gains or losses. Duplications result in a higher-intensity fluorescence relative to control, and deletions result in a lower-intensity fluorescence. CGH is most useful in the detection of relatively large duplications or deletions.⁷⁵

In SNP arrays only the patient DNA is labeled, denatured, and hybridized to an array containing probes with known SNPs. Again, the signal intensity is used to determine the copy number. SNP arrays are able to detect runs of homozygosity that can indicate uniparental disomy or consanguinity.⁷⁶ Because each of these methods has both advantages and disadvantages, array platforms have been developed that contain both types of sequences: SNPs and larger clones used in CGH. This provides a more uniform coverage over the entire genome. In certain situations, arrays are replacing or used as an adjunct to conventional karyotyping and fluorescence in situ hybridization (FISH) (Chapter 30). Arrays are useful to detect copy number variants but do not detect balanced translocations.⁷⁷⁻⁷⁸

PATHOGEN DETECTION AND INFECTIOUS DISEASE LOAD

Box 31-4 contains a listing of hematologically important pathogens detected by molecular methods. Real-time quantitative PCR can detect and quantitate a number of blood-borne viruses: hepatitis B and C viruses, human papillomavirus,

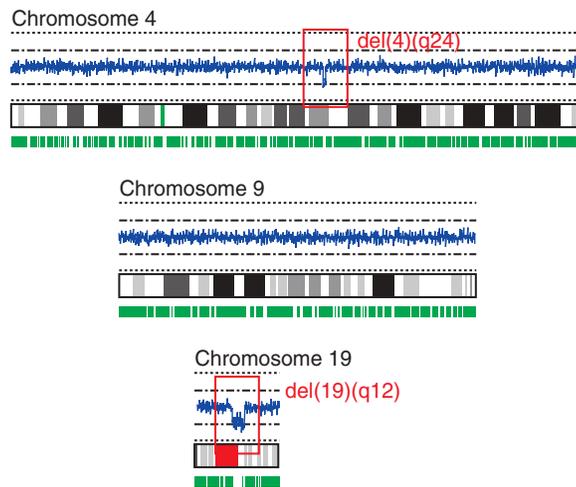
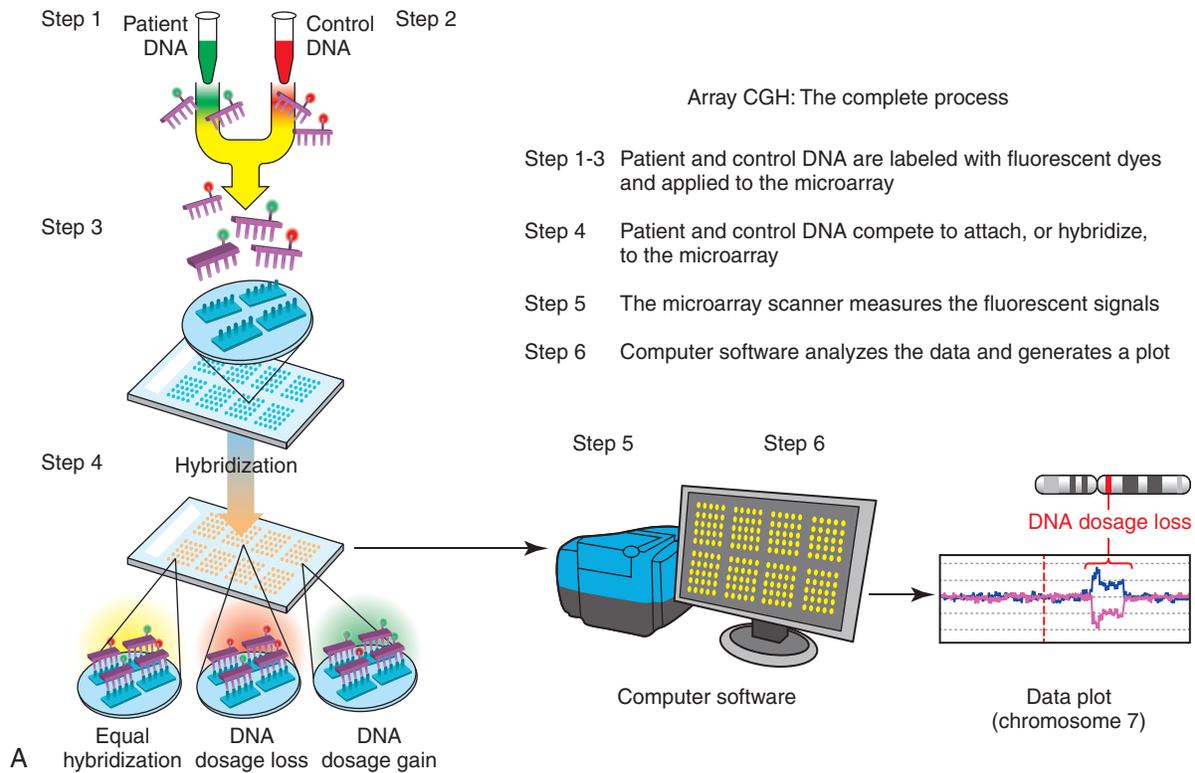


Figure 31-23 Chromosomal microarrays. **A**, Diagram of the procedure for microarray-based comparative genomic hybridization (aCGH). **B**, An example of a single nucleotide polymorphism array (SNP-A) karyogram from a patient with a secondary acute myeloid leukemia with microdeletions on chromosomes 4 and 19 and normal chromosome 9. (© 2008 SLACK, Inc. Modified from Shaffer L. G. & Bejjani B. A. Using microarray-based molecular cytogenetic methods to identify chromosome abnormalities. *Pediatric Annals* 38, 440-447 (2009) doi: 10.3928/00904481-20090723-08.)

CMV, Epstein-Barr virus, and HIV.⁷⁹ Human bacterial pathogens such as β -hemolytic streptococcus from throat swabs, anaerobes from wound swabs, and bacteria from urine or other body fluids can be detected within hours of collection. Antibacterial therapy can be initiated based upon the rapid results of molecular susceptibility testing. Real-time quantitative PCR is the reference method for detection and quantification of methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant enterococcus, and opportunistic *Clostridium difficile*. Molecular diagnostic techniques are effective in identifying

and monitoring malarial and other blood-borne parasites. The challenge to primer and probe developers is to select sequences that are specific enough to avoid false positives caused by non-pathogenic strains, sensitive enough to positively identify infectious strains, and flexible enough to remain effective as pathogenic microorganisms mutate and evolve. There are currently multiple FDA approved assays for the detection of viral and bacterial pathogens. A current listing of these tests can be found in the test directory on the Association for Molecular Pathology web site (amp.org).

Clinical relevance is important when assessing infectious disease using molecular techniques. These methods allow millions of copies to be generated from a single DNA or RNA sequence from a microorganism or virus. Theoretically, the presence of a single organism can lead to a positive test result, but a single organism may not be clinically relevant. Standard curves of template number are crucial to data interpretation. Also, because DNA survives the organism, a positive result on a test for a given sequence does not guarantee that the organism was viable at the time of sampling.

CURRENT DEVELOPMENTS

Molecular diagnostics is a rapidly growing area of the clinical laboratory, and the technology continues to develop. It promises to revolutionize laboratory techniques in all disciplines, and the technologies of genomics are being extended to proteomics (the molecular analysis of proteins) and metabolomics (the molecular analysis of metabolism). Methods continue to be automated and miniaturized, providing ever greater sensitivity and reliability coupled with short turnaround time and technical simplification. In many situations, assays are moving from single analyte assays to multiplex assays detecting panels of analytes. In the case of leukemias such as AML, mutations in multiple genes are incorporated into the WHO guidelines.⁸⁰ Methods such as the SNaPshot technique described previously are being applied to detect multiple mutations simultaneously.

Another technique being applied to the detection of mutation panels is Matrix-assisted laser desorption/ionization—time of flight (MALDI-TOF) mass spectrometry. This methodology uses PCR coupled to a single-base extension reaction that adds labeled nucleotides so that the extension products containing different mutations have different masses. These reactions are also multiplexed to increase throughput, detecting hundreds of mutations in a single panel assay.⁸¹

Digital PCR (ddPCR) is a technique with very high sensitivity that can be used to detect resistance mutations to tyrosine kinase inhibitors used to treat CML (such as the T315I resistance mutation in the *BCR-ABL1* gene)⁸² (Chapter 33) or to quantify virus copy number.⁸³ This technique uses various methods—for example, a droplet generator to create nanoliter droplets that partition template molecules, which are then amplified by PCR. The amplicons are detected by fluorescence and either read by a droplet reader or other detection mecha-

nism. For translocation detection, wells containing an amplified housekeeping gene, translocation product, or both are then quantitated. This method is extremely sensitive, detecting a few molecules per sample, and can be applied to both DNA and RNA applications.

NGS will continue to play an increasingly important role in molecular diagnostics. In addition to sequencing panels of genes, this technology has been used to sequence whole genomes, exomes (the coding exons), as well as RNA sequencing (RNAseq)^{84,85} (Figure 31-24). This technology is also being applied to the determination of the epigenome⁸⁶—modifications such as methylation that affect gene regulation and expression.

Small microRNAs (miR; ~20 nucleotides long) that were once thought to be insignificant are evolving as biomarkers for the progression of hematologic malignancies. The dysregulation of miRs affects normal hematopoiesis, and their atypical expression is beginning to be established in T and B cell leukemias and lymphomas.⁸⁷ These miRs target genes in the 3' UTR (3' untranslated region) and are hypothesized to inhibit the translation of mRNA to proteins.⁸⁸ These miRs can be detected by many molecular-based techniques such as PCR, NGS, and microarray technology. Their clinical role in hematologic cancer therapy as a prognostic marker is now starting to be recognized. A link between p53 expression and possible therapeutic efficacy is shown with miR-181a/b dysregulation in chronic lymphocytic leukemia (CLL) patients in a recent study.⁸⁹ Another example is a report by Seca and colleagues⁹⁰ that lists many functions of miR-21, including the upregulation of the BCR-ABL1 protein and induction of chemoresistance. An overexpression of miR-21 has been demonstrated in patients who are fludarabine nonresponders.⁹¹ The overexpression of plasma miR-155 is correlated to the identification of B-CLL in patients,⁹² and association of miR-21 is established with drug resistance in plasma cell myeloma.⁹³ The listed studies demonstrate that the ability to measure the expression of miR has expanded the repertoire of diagnostic, prognostic, and therapeutic efficacy markers in hematologic malignancies.

The future molecular technologies will increase the efficiency and sensitivity for detection of all types of genome alterations, including point mutations, insertion and deletion mutations, copy number variants, and chromosome rearrangements. It will facilitate the discovery of new chromosome rearrangements as well as the diagnosis of microbial infections. It will result in refined classification and improved treatment of hematologic diseases.

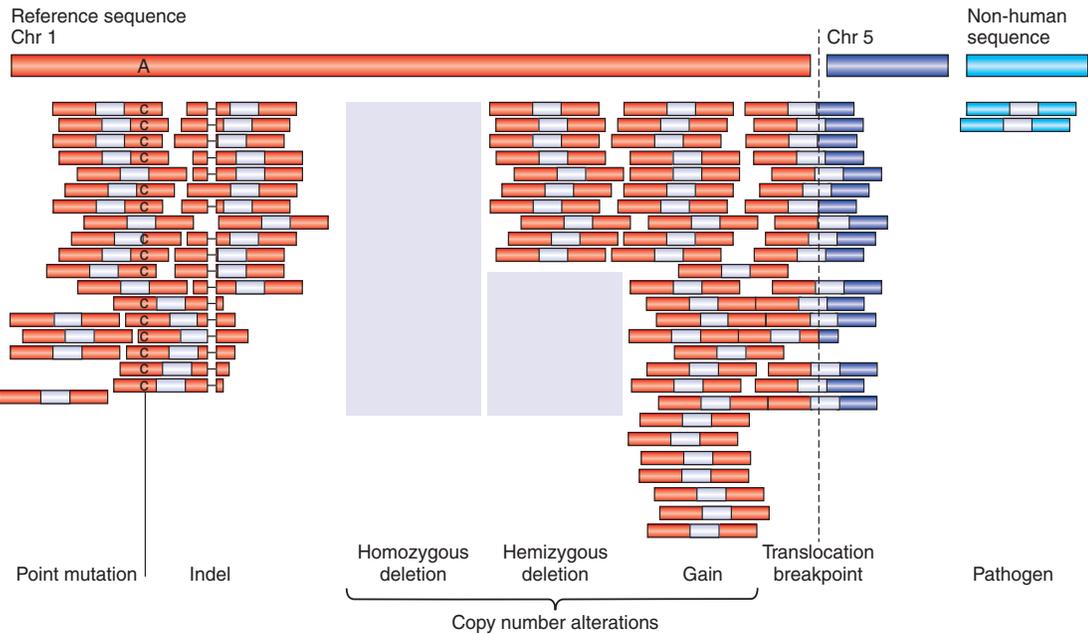
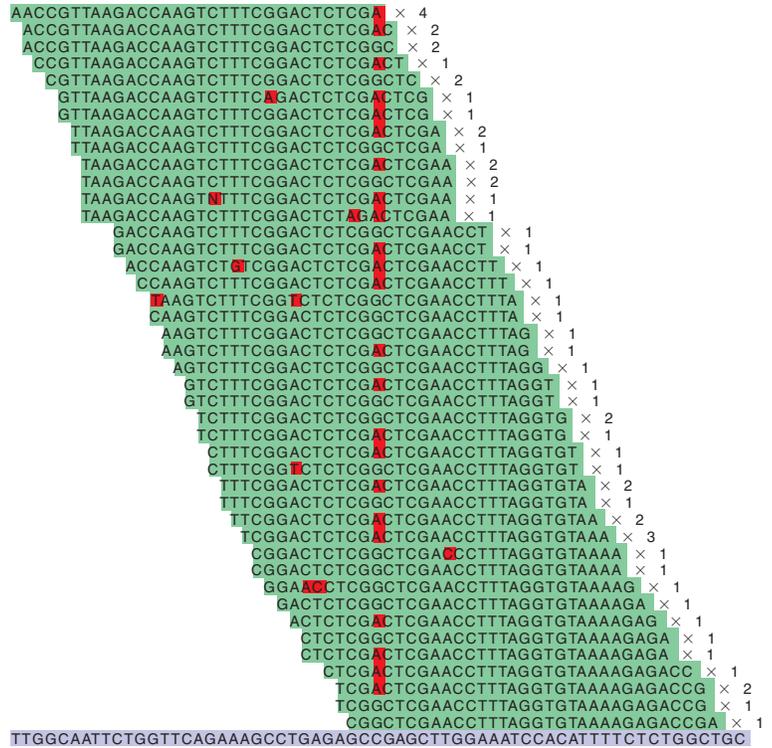
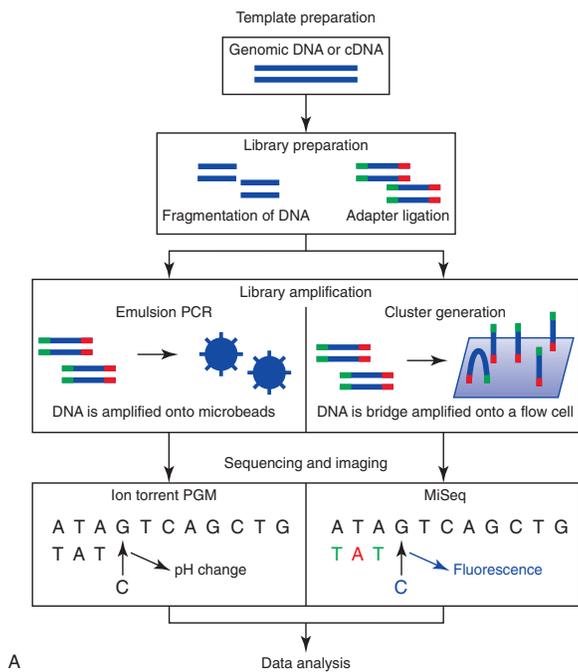


Figure 31-24 **A**, Diagram of the procedure for next-generation sequencing (NGS) using the two most common technologies. **B**, An illustration of a number of NGS reads for a 32-nucleotide sequence aligned with the genomic reference sequence in blue on the bottom (sequence mismatches are in red). The center of the alignment shows a variant present in the heterozygous state. **C**, Sequenced fragments are depicted as bars with colored tips representing the sequenced ends and the unsequenced portion of the fragment in gray. Reads are aligned to the reference genome (for example, mostly chromosome 1 in this example). The colors of the sequenced ends show where they align. Different types of genomic alterations can be detected. From left to right, point mutations (in this example, A to C) and small insertions and deletions (indels) (in this example, a deletion shown by a dashed line) are detected by identifying multiple reads that show non-reference sequence; changes in sequencing depth (relative to a normal control) are used to identify copy number changes (shaded boxes represent absent or decreased reads in a tumor sample); paired-ends that map to different genomic loci (in this case, chromosome 5) are evidence of rearrangements; and sequences that map to nonhuman sequences are evidence for the potential presence of genomic material from pathogens. (**A** from Grada A, Weinbrecht K: Next-generation sequencing: methodology and application. *Journal of Investigative Dermatology* 133, 248-251, 2013; **B** from Almomani R, van der Heijden J, Ariyurek Y, et al: Experiences with array-based sequence capture; toward clinical applications. *European Journal of Human Genetics* 19:50-55, 2011; **C** from Meyerson M, Gabriel S, Getz G: Advances in understanding cancer genomes through second generation sequencing. *Nature Reviews Genetics* 11: 685-696, 2010.)

SUMMARY

- DNA directs cell function as described by the *central dogma*. DNA retains the genetic code and reproduces itself through *replication*.
- The genetic code is *transcribed* from DNA to mRNA. mRNA consists of coding exons and noncoding introns that are excised after transcription. The processed mRNA then transports the code from the nucleus to the cytoplasm, where it is *translated* by the cytoplasmic ribosomes. Translation depends on tRNA, small RNA molecules designed to transport and add amino acid to growing peptide chains in cytoplasmic ribosomes.
- DNA consists of a five-carbon sugar (deoxyribose), a phosphate group, and a nitrogenous base. The bases are either purines or pyrimidines. The purines in DNA are adenine (A) and guanine (G). The pyrimidines in DNA are thymine (T) and cytosine (C).
- DNA is a double-stranded molecule held together by hydrogen bonding between the bases, A to T and G to C. Heat denatures double-stranded DNA by breaking the hydrogen bonds to produce single-stranded DNA. DNA strands are antiparallel.
- RNA is a single-stranded molecule that contains the sugar ribose instead of the deoxyribose found in DNA and the pyrimidine uracil in place of thymine.
- RNA polymerase recognizes a sequence of deoxyribonucleotides called the *promoter* within DNA. RNA polymerase separates the DNA strands and begins adding ribonucleotides, forming an initial RNA transcript consisting of introns and exons.
- Proteins function as structural components of the cell, as enzymes involved in metabolism or regulation, as receptors to regulate cellular functions, or as antibodies for the immune system. Mutation within a gene ultimately alters the protein produced, which often affects the function of the protein.
- Five areas of hematopathologic molecular testing include detection of mutations, gene rearrangements, and chromosomal abnormalities for diagnosis and prognosis of hematologic malignancies; detection and quantification of minimal residual disease to monitor treatment of hematologic malignancies; detection of mutations in inherited hematologic disorders; pharmacogenetic testing to detect genetic variation affecting certain drug therapies; and identification of hematologically important infectious diseases.
- Peripheral blood, bone marrow, tissue biopsy samples (both fresh and formalin fixed paraffin-embedded), fine-needle aspirates, body fluids, saliva, and cheek swabs are specimens used for DNA and RNA isolation.
- DNA is amplified by in vitro endpoint polymerase chain reaction (PCR) and real-time PCR. RNA targets can be amplified by PCR by first converting the RNA target to complementary DNA or cDNA.
- In endpoint PCR, amplified DNA is detected by electrophoresis (slab gel or automated capillary gel electrophoresis) or cleavage-based signal amplification (Invader) technology. Endpoint PCR can also be combined with restriction enzyme digestion of the amplicons, followed by detection of the restriction fragments by one of the methods mentioned above. In real-time PCR, the amplicons are detected during the PCR cycles by fluorescence detection.
- Real-time PCR can be used qualitatively to determine the presence or absence of a target or can quantify the copy number of a target DNA or RNA. PCR can be used to amplify RNA targets by first converting them to cDNA with reverse transcriptase activity.
- The dideoxy chain termination (Sanger) method for DNA sequencing is based on the principle that synthesis of a DNA polymer is terminated upon incorporation of a dideoxynucleotide. The target DNA template is amplified over a number of cycles, which produces a series of DNA fragments that terminate at each successive base with each fragment differing in length by one nucleotide. DNA fragments are detected by labeling either the dideoxynucleotide or the primer with a fluorescent dye. Other methods for DNA sequencing include pyrosequencing and next-generation sequencing (NGS).
- Chromosomal microarrays measure gains and losses of genomic DNA. They provide much greater sensitivity in detecting small genomic changes compared to conventional karyotyping. There are two types: microarray-based comparative genomic hybridization (aCGH) and single nucleotide polymorphism array (SNP-A) karyotyping. CGH requires the use of a control DNA.
- Molecular testing permits clinicians to make more accurate diagnostic, therapeutic, and prognostic decisions. It also allows a more sensitive assessment of minimal residual disease and therapeutic efficacy, resulting in better patient management.

Now that you have completed this chapter, go back and read again the case study at the beginning and respond to the questions presented.

Review Questions

Answers can be found in the Appendix.

1. If the DNA nucleotide sequence is 5'-ATTAGC-3', then the mRNA sequence transcribed from this template is:
 - a. 5'-GCUAAU-3'
 - b. 5'-AUUAGC-3'
 - c. 5'-TAATCG-3'
 - d. 5'-UAAUCG-3'
2. Cells with damaged DNA and mutated or nonfunctioning cell cycle regulatory proteins:
 - a. Are arrested in G₁ and the DNA is repaired
 - b. Continue to divide, which leads to tumor progression
 - c. Divide normally, producing identical daughter cells
 - d. Go through apoptosis

3. To start DNA replication, DNA polymerase requires an available 3' hydroxyl group found on the:
 - a. Leading strand
 - b. mRNA
 - c. Parent strand
 - d. Primer
4. Ligase joins Okazaki fragments of the:
 - a. 5'-to-3' template strand
 - b. Lagging strand
 - c. Leading strand
 - d. Primer fragments
5. A 40-year-old patient enters the hospital with a rare form of cancer caused by faulty cell division regulation. This cancer localized in the patient's spleen. An ambitious laboratory developed a molecular test to verify the type of cancer present. This molecular test would require patient samples taken from which two tissues?
 - a. Abnormal growths found on the skin and in the bone marrow
 - b. Normal splenic tissue and cancerous tissue
 - c. Cancerous tissue in spleen and bone marrow
 - d. Peripheral blood and cancerous tissue in the spleen
6. One main difference between PCR and reverse transcriptase PCR is that:
 - a. PCR requires primers
 - b. PCR uses reverse transcriptase to elongate the primers
 - c. Reverse transcriptase PCR forms millions of cDNA fragments
 - d. Reverse transcriptase PCR requires ligase to amplify the target DNA
7. Which one of the following statements about gel electrophoresis is *false*?
 - a. The gel is oriented in the chamber with the wells at the positive terminal.
 - b. A buffer solution is required to maintain the electrical current.
 - c. The matrix of a polyacrylamide gel is tighter than that of an agarose gel.
 - d. The larger DNA fragments will be closest to the wells of the gel.
8. Autoradiography of DNA is the:
 - a. Detection of radioactive or chemoluminescent oligonucleotides
 - b. Exposure of the gel to UV light
 - c. Transfer of DNA to a nitrocellulose filter
 - d. Use of ethidium bromide to visualize the DNA banding pattern
9. One major difference between endpoint PCR and real-time PCR is that:
 - a. Endpoint PCR requires thermostable DNA polymerase, deoxynucleotides, and primers
 - b. Endpoint PCR requires a separate step to detect the amplicons formed in the reaction
 - c. Real-time PCR uses capillary gel electrophoresis to detect amplicons during PCR cycling
 - d. Real-time PCR detects and quantifies amplicons using cleavage-based signal amplification
10. Which of the following statements about minimal residual disease is *true*?
 - a. Clinical remission of hematologic cancers is determined by molecular techniques such as PCR and flow cytometry.
 - b. Real-time quantitative PCR-determined copy number of *BCR/ABL1* transcripts will always be lower in molecular remission than in clinical remission.
 - c. Qualitative PCR that uses a known copy number of a target sequence is of use in determining minimal residual disease levels.
 - d. Minimal residual disease assessment can aid physicians in making treatment decisions but does not yet offer insights into prognosis.

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Flow Cytometric Analysis in Hematologic Disorders

Magdalena Czader

OUTLINE

**Specimen Processing
Flow Cytometry: Principle
and Instrumentation
Pattern Recognition Ap-
proach to Analysis of
Flow Cytometric Data**
*Concept of Gating
Analysis of Flow Cytometric
Data*

**Cell Populations Identified
by Flow Cytometry**
*Granulocytic Lineage
Monocytic Lineage
Erythroid Lineage
Megakaryocytic Lineage
Lymphoid Lineage*

**Flow Cytometric Analysis
of Myeloid Disorders
(Acute Myeloid Leuke-
mias and Chronic My-
eloid Neoplasms)**
*Acute Myeloid Leukemias
with Recurrent Cyto-
genetic Abnormalities
Acute Myeloid Leukemias
Not Otherwise Specified
Myeloproliferative Neo-
plasms and Myelodysplas-
tic Syndromes*

**Flow Cytometric Analysis
of Lymphoid Neoplasms
(Lymphoblastic Leuke-
mia/Lymphoma and Ma-
ture Lymphoid Neo-
plasms)**
*B Lymphoblastic Leukemia/
Lymphoma
T Lymphoblastic Leukemia/
Lymphoma
Mature Lymphoid Neo-
plasms*

**Other Applications of Flow
Cytometry Beyond Im-**

OBJECTIVES

After completion of this chapter, the reader will be able to:

1. Describe the technique of flow cytometry, including specimen selection and preparation, instrumentation, data collection, and a design of an antibody panel.
2. Discuss the pattern recognition approach to analysis of flow cytometric data for diagnosis and follow-up of hematologic malignancies.
3. Identify basic cell populations defined by flow cytometric parameters.
4. Recognize the key immunophenotypic features of normal bone marrow, peripheral blood, and lymph node tissue, and specimens from patients with acute leukemia or lymphoma.
5. Discuss novel applications of flow cytometry beyond the immunophenotyping of hematologic malignancies.

CASE STUDIES

After studying the material in this chapter, the reader should be able to respond to the following case studies:

Case 1

A 58-year-old man had a 5-month history of extensive right cervical lymphadenopathy and night sweats. His complete blood count (CBC) results were within normal limits. Physical examination showed additional bilateral axillary lymphadenopathy. The cervical lymph node was excised. Histologic examination revealed nodular architecture with predominantly medium-sized lymphoid cells with irregular nuclear outlines. Flow cytometric data are presented in [Figure 32-1](#).

1. What cell subpopulation predominates on the forward scatter (FS)/side scatter (SS) scattergram?
2. List antigens positive in this population.
3. Does the pattern of light chain expression support the diagnosis of lymphoma?

Case 2

A 3-year-old girl was brought to the physician because of fatigue and fevers. The CBC revealed a WBC count of $3 \times 10^9/L$, HGB level of 8.3 g/dL, and platelet count of $32 \times 10^9/L$. Review of the peripheral blood film showed rare undifferentiated blasts with occasional cytoplasmic blebs. No granules or Auer rods were identified. Bone marrow examination showed a marked increase in blasts (79%) and decreased trilineage hematopoiesis. Flow cytometric analysis was performed. In addition to the markers shown in [Figure 32-2](#), the population of interest was positive for CD34, CD33, CD41, and HLA-DR.

1. What abnormal features are observed on the CD45/SS scattergram?
2. What is the most likely diagnosis considering the constellation of markers expressed by the predominant population?

Continued

CASE STUDIES—cont'd

After studying the material in this chapter, the reader should be able to respond to the following case studies:

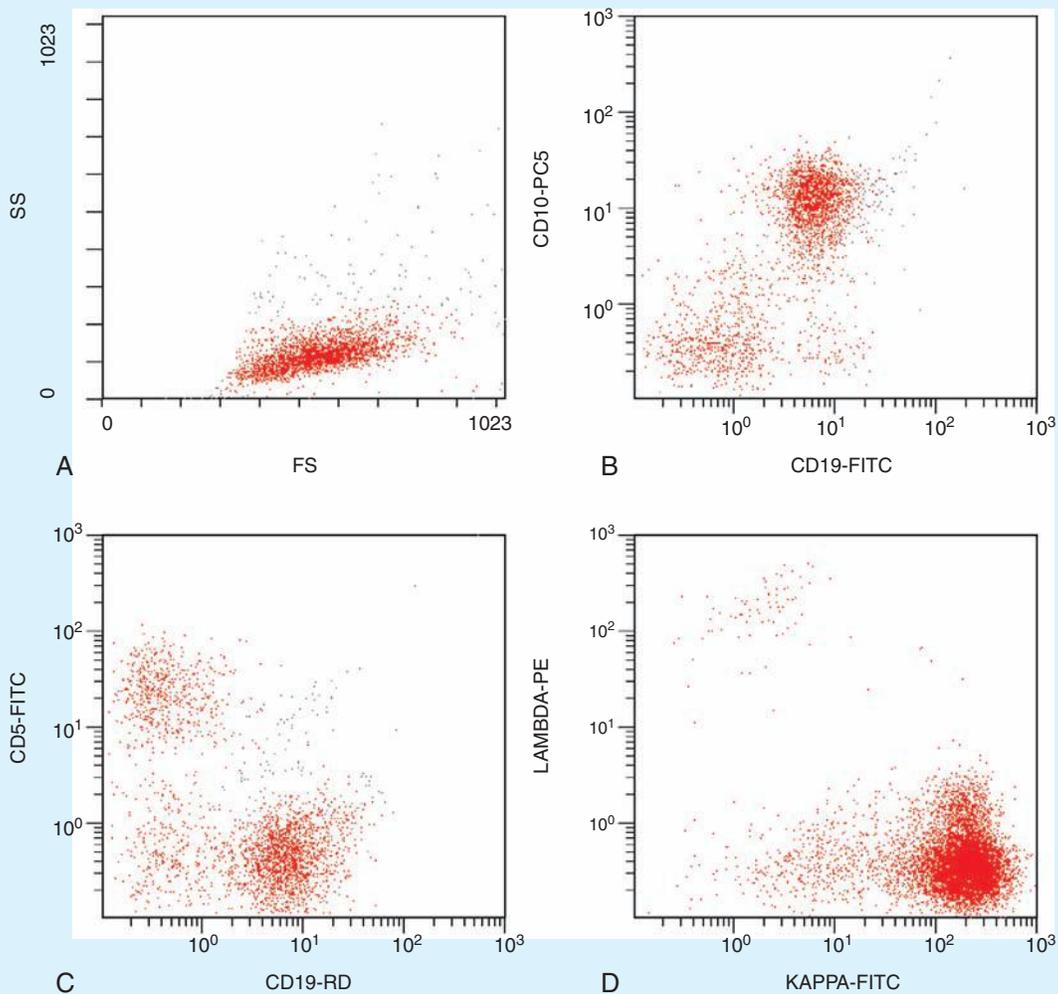


Figure 32-1 Scattergrams showing immunophenotypic features of lymphoid cells from the patient in Case 1. FITC, fluorescein isothiocyanate; FS, Forward scatter; RD, rhodamine; SS, side scatter.

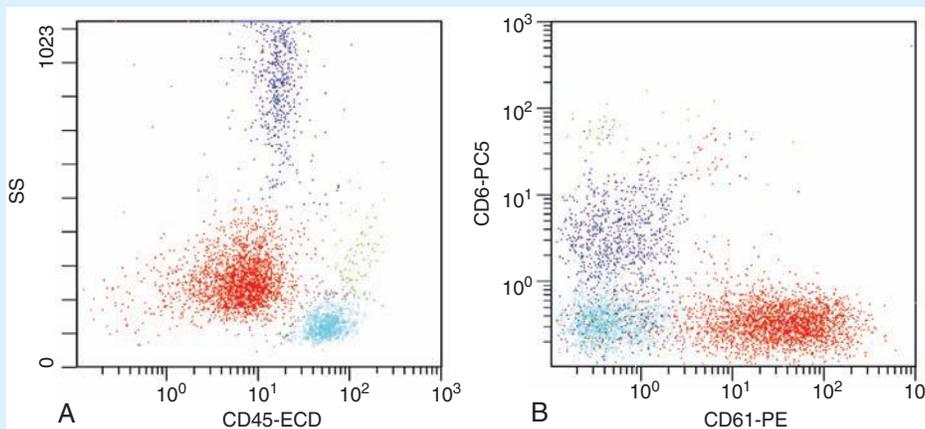


Figure 32-2 Predominant bone marrow population for the patient in Case 2. ECD, phycoerythrin-Texas Red; PE, phycoerythrin; SS, Side scatter.

Flow cytometry was originally designed to measure physical properties of cells based on their ability to deflect light. Over the years, it has evolved to include detection of fluorescent signals emitted by dyes bound directly to specific molecules or attached to proteins through monoclonal antibodies. The development of monoclonal antibodies is the most significant factor contributing to today's broad application of flow cytometry. Although the term *flow cytometry* implies the measurement of a cell, this technique is applied successfully to study other particles, including chromosomes, microorganisms, and proteins. The main advantage of flow cytometry over other techniques is its ability to rapidly and simultaneously analyze multiple parameters in a large number of cells. When one adds the capability of identifying and quantifying rare-event cells in a heterogeneous cell population, the value of flow cytometry to clinical hematology becomes obvious. Currently, this technique not only is applied to analysis of cell lineage in acute leukemia or a detection of clonality in lymphoid populations but also makes it possible to discern abnormal populations in chronic myeloid neoplasms, quantitate minimal residual disease, and monitor immunodeficiency states. Immunophenotypes that originally were used to supplement morphologic classification frequently correlate with specific cytogenetic or molecular abnormalities. According to the classification of hematopoietic neoplasms recommended by the World Health Organization,¹ one no longer can rely solely on morphology for a diagnosis of hematologic malignancies. Current diagnostic algorithms integrate morphologic, immunophenotypic, and genotypic information. This approach emphasizes the central role that flow cytometry plays in the hematopathology laboratory.

The focus of this chapter is on the use of flow cytometry in a routine hematopathology laboratory. The chapter follows a "life" of a flow cytometric specimen that starts with specimen processing and ends with a final diagnosis. The discussion is divided into preanalytical (specimen processing), analytical (flow cytometric instrumentation and analysis), and postanalytical (immunophenotypic features of hematopoietic disorders) sections.

SPECIMEN PROCESSING

Flow cytometric analysis is particularly useful in diagnosing hematologic malignancies. The specimens most commonly analyzed are bone marrow, peripheral blood, and lymphoid tissues. In addition, immunophenotyping is often performed on body cavity fluids and solid tissues when they are suspected to harbor a hematologic malignancy.²

Prolonged transport or transport under inappropriate conditions may render a specimen unsuitable for analysis. Peripheral blood and bone marrow specimens should be processed within 24 to 48 hours from the time of collection. Certain specimens, such as body cavity fluids or samples from neoplasms with a high proliferative activity, may require even more rapid processing.

When cells are suspended in a fluid, as in peripheral blood and bone marrow, minimal sample preparation is required. These specimens are collected into a tube or container with an anticoagulant, preferably heparin, and are transported to a flow cytometry laboratory at room temperature. Bone marrow

biopsy specimens and solid tissue specimens, including core biopsy samples, are submitted in culture media to maintain viability or on saline-moistened gauze. Tissue fragments are mechanically dissociated to yield a cell suspension, usually by mincing with a scalpel.

To obtain a pure population of nucleated cells, red blood cells (RBCs) are lysed. The analytical process depends on cellularity and viability of a specimen; both are routinely assessed before a sample is stained. Cell count can be obtained using automated cell counters or flow cytometry. A specimen is stained with propidium iodide or 7-amino actinomycin to test viability. A cytocentrifuge slide (Chapter 18) is prepared for a morphologic inspection of a cell suspension.

As soon as these steps are completed, a sample is stained with a cocktail of fluorochrome-conjugated monoclonal antibodies. The analysis of intracytoplasmic markers requires an additional fixation and permeabilization step to allow antibodies to pass through a cell membrane. A predetermined panel of antibodies may be used to detect membrane-bound and intracellular markers. Simultaneous analysis of multiple markers, known as *multicolor* or *multiparameter flow cytometry*, has numerous advantages. It facilitates visualization of antigen expression and maturation patterns, which are often disturbed in hematopoietic malignancies. In addition, regardless of a complexity of a specimen, analysis can be accomplished using few tubes and with a lower total number of cells, which saves reagents, time, and data storage. There is no consensus on the standardized panel of antibodies to be used in routine flow cytometric evaluation. The U.S.-Canadian Consensus Project in Leukemia/Lymphoma Immunophenotyping recommends the comprehensive approach with multiple markers for myeloid and lymphoid lineage.³ Selected markers commonly analyzed by flow cytometry are presented in Table 32-1.

FLOW CYTOMETRY: PRINCIPLE AND INSTRUMENTATION

The most significant discovery that led to the advancement of flow cytometry and its subsequent widespread application in clinical practice was the development of monoclonal antibodies.⁴ In the original *hybridoma* experiments, lymphocytes with predetermined antibody specificity were co-cultured with a myeloma cell line to form immortalized hybrid cells producing specific monoclonal antibodies. For this discovery, which not only fueled the development of flow cytometry but also had innumerable research and, more recently, clinical applications, Köhler and Milstein received a Nobel Prize in 1984. Over the years, numerous antibodies were produced and tested for their lineage specificity. Categorization of these antibodies and associated antigens is accomplished through workshops on human leukocyte differentiation antigens that have been held regularly since 1982. These workshops provide a forum for reporting new antigens and antibodies and define a cluster of antibodies recognizing the same antigen, called *cluster of differentiation* (CD) (Table 32-2; see also Table 32-1). Consecutive numbers are assigned to each new reported antigen. The Ninth International Conference on Human Leukocyte Differentiation Antigens brought to over 350 the total number of antigens characterized.⁵

TABLE 32-1 Lineage-Associated Markers Commonly Analyzed in Routine Flow Cytometry

Lineage	Markers
Immature	CD34
	CD117
	Terminal deoxynucleotidyl transferase
Granulocytic/monocytic	CD33
	CD13
	CD15
	CD14
Erythroid	CD71
	Glycophorin A
Megakaryocytic	CD41
	CD42
	CD61
B lymphocytes	CD19
	CD20
	CD22
	κ Light chain
	λ Light chain
T lymphocytes	CD2
	CD3
	CD4
	CD5
	CD7
	CD8

Monoclonal antibodies have various applications, including immunohistochemistry, immunofluorescence, and Western blot. These methods study cellular proteins in fixed tissues or in cellular extracts; however, they do not provide the ability to examine antigens in their native state and cannot decipher composite cell populations with a complex antigen makeup. In contrast, flow cytometry can define antigen expression on numerous viable cells. Currently, 17 antigens can be detected simultaneously on an individual cell.⁶ This is accomplished by the conjugation of monoclonal antibodies to a variety of fluorochromes that can be detected directly by a flow cytometer. In a flow cytometer, particles are suspended in fluid and pass one by one in front of a light source. As particles are illuminated, they emit fluorescent signals registered by detectors. These results are later converted to digital output and analyzed using flow cytometry software. The flow cytometer consists of fluidics, a light source (laser), a detection system, and a computer. A brief discussion of these basic components is presented.

To be analyzed individually, cells must pass separately, one by one, through the illumination and detection system of a flow cytometer. This passage is accomplished by injecting a cell suspension into a stream of sheath fluid. This technique, called *hydrodynamic focusing*, creates a central core of individually aligned cells surrounded by a sheath fluid (Figure 32-3). The central alignment is essential for consistent illumination of cells as they pass before a laser light source.

A laser is composed of a tube filled with gas, most commonly argon or helium-neon, and a power supply. Current is

TABLE 32-2 Hematolymphoid Antigens Commonly Used in Clinical Flow Cytometry

Cluster of Differentiation	Function	Cellular Expression
CD1a	T cell development	Precursor T cells
CD2	T cell activation	Precursor and mature T cells, NK cells
CD3	Antigen recognition	Precursor and mature T cells
CD4	Co-receptor for HLA class II	Precursor T cells, helper T cells, monocytes
CD5	T cell signaling	Precursor and mature T cells, subset of B cells
CD7	T cell activation	Precursor and mature T cells, NK cells
CD8	Coreceptor for HLA class I	Precursor T cells, suppressor/cytotoxic T cells, subset of NK cells
CD10	B cell regulation	Precursor B cells, germinal center B cells, granulocytes
CD11b	Cell adhesion	Granulocytic and monocytic lineage, NK cells
CD13	Unknown	Granulocytic and monocytic lineage
CD14	Monocyte activation	Mature monocytes
CD15	Ligand for selectins	Granulocytic and monocytic lineage
CD16	Low-affinity IgG Fc receptor	Granulocytic and monocytic lineage, NK cells
CD18	Cell adhesion and signaling	Granulocytic and monocytic lineage
CD19	B cell activation	Precursor and mature B cells
CD20	B cell activation	Precursor and mature B cells
CD22	B cell activation and adhesion	Precursor and mature B cells
CD31	Cell adhesion	Megakaryocytes, platelets, leukocytes
CD33	Cell proliferation and survival	Granulocytic and monocytic lineage
CD34	Cell adhesion	Hematopoietic stem cells
CD36	Cell adhesion	Megakaryocytes, platelets, erythroid precursors, monocytes
CD38	Cell activation and proliferation	Hematopoietic cells, including activated lymphocytes and plasma cells
CD41	Cell adhesion	Megakaryocytes, platelets
CD42b	Receptor for von Willebrand factor	Megakaryocytes, platelets
CD45	T and B cell receptor activation	Hematopoietic cells
CD56	Cell adhesion	NK cells, subset of T cells

TABLE 32-2 Hematolymphoid Antigens Commonly Used in Clinical Flow Cytometry—cont'd

Cluster of Differentiation	Function	Cellular Expression
CD61	Cell adhesion	Megakaryocytes, platelets
CD62P	Homing	Platelets
CD63	Cell development, activation, growth, and motility	Platelets
CD64	High-affinity IgG Fc receptor	Granulocytic and monocytic lineage
CD71	Iron uptake	High density on erythroid precursors, low to intermediate density on other proliferating cells
CD79a	B cell receptor signal transduction	Precursor and mature B cells
CD117	Stem cell factor receptor	Hematopoietic stem cells, mast cells

Ig, Immunoglobulin; NK, natural killer.

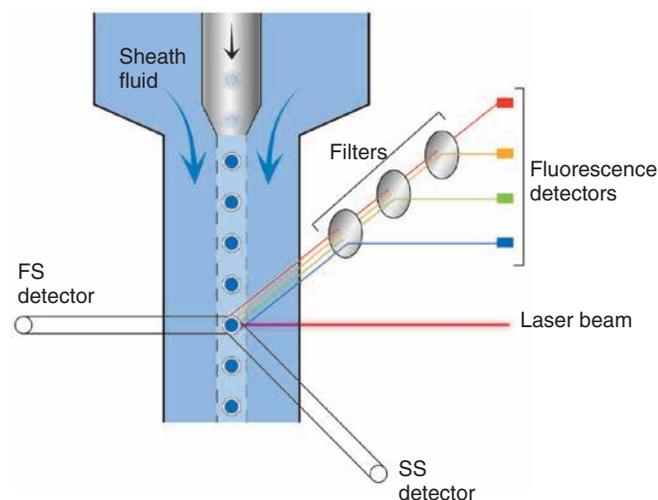


Figure 32-3 Diagram of a flow cytometer. As cells are injected into pressurized sheath fluid, they are positioned in the center of the stream and one by one exposed to the laser light. Forward scatter (FS) and side scatter (SS) are collected by separate detectors.

applied to the gas to raise electrons of a gas to an excited state. When electrons return to a ground state, they emit photons of light. Through an amplification system, a strong beam of light with light waves of identical direction, polarization plane, and wavelength is produced. This narrow coherent beam of light is used to illuminate individual cells, each stained with antibodies conjugated to specific fluorochromes.

After absorption of laser light, the electrons of fluorochromes are raised from a ground state to a higher energy state (Figure 32-4). The return to the original ground level is accompanied by a loss of energy, emitted as light of a specific wavelength. Flow cytometers are equipped with several photodetectors, each specific for light of a unique color (wavelength). The fluorescence from an individual cell is partitioned into its different wavelengths through a series of filters (dichroic mirrors) and directed to the corresponding photodetector. Fluorescent signals derived from different fluorochromes attached to particular antibodies are registered separately.

In addition to fluorescence, scatter signals are recorded. The detector situated directly in line with the illuminating laser beam measures forward scatter (FS or FSC), which is proportional to particle volume or size. A photodetector located to the side measures side scatter (SS or SSC), which reflects surface complexity and internal structures such as granules and

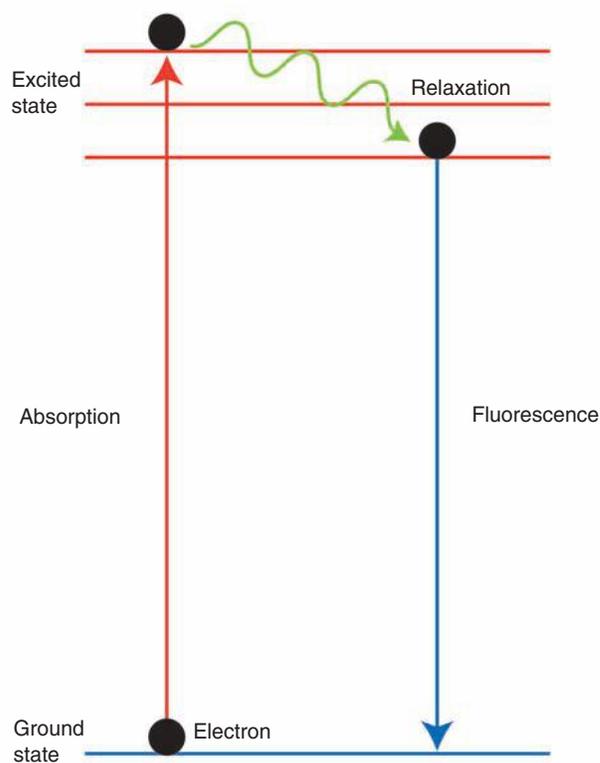


Figure 32-4 Jablonski diagram showing a principle of fluorescence. When electrons absorb energy, they are raised to the excited state. Subsequently, on their return to ground state, the absorbed energy is emitted in a form of fluorescence.

vacuoles. FS, SS, and fluorescence are displayed simultaneously on the instrument screen and registered by the computer system.

PATTERN RECOGNITION APPROACH TO ANALYSIS OF FLOW CYTOMETRIC DATA

Concept of Gating

Cell populations with similar physical properties such as size, cytoplasmic complexity, and expression of a specific antigen form *clusters* on data displays generated by flow cytometers. A *gate* is an electronic boundary an operator uses to delineate cell clusters. Thus, gating is a process of selecting, with a cursor or computer mouse, a population of interest as defined by one or more flow cytometric parameters.

Gating can be applied at the time of data acquisition (live gate) or at the time of analysis. For diagnostic purposes, data

are collected ungated; that is, all events detected by the flow cytometer are recorded. This allows comprehensive testing and retention of positive and negative internal controls. In addition, unexpected abnormal populations are detected. Gating is most commonly applied after a specimen is run through a flow cytometer, when a target population is already known. In contrast, live gating focuses on the acquisition of data for a specific cell population as defined by flow cytometric parameters. For example, one can collect data only on CD19⁺ B cells to facilitate detection of a small population of monoclonal B cells.

Analysis of Flow Cytometric Data

As with microscopic examination, an evaluation of flow cytometric data is based on the inspection of visual patterns. First, the data are scanned to detect abnormal populations. Subsequent analysis focuses on the antigenic properties of these abnormal cells.

Analysis begins with inspection of dot plots presenting cell size, cytoplasmic complexity, and expression of pan-hematopoietic antigen CD45. As in microscopic examination at low magnification, an operator detects specific cell populations based on their physical properties (Figure 32-5). The identification of particular populations can be confirmed and further resolved on the scattergram of CD45 antigen density and SS (Figure 32-6). This display also provides information on the relative proportion of specific cell populations in the flow cytometric sample. Lymphocytes show the highest density of CD45, with approximately 10% of the cell membrane occupied by this antigen. Granulocytic series show intermediate CD45 density; late erythroid precursors and megakaryocytes are negative for CD45. The CD45/SS display is particularly useful for detection of blasts, which overlap with lymphocytes, monocytes, or both on the FS/SS display.^{7,8}

The FS/SS and CD45/SS displays allow the initial identification of the target population. Further analysis focuses on patterns of antigen expression, including qualitative data (antigen presence or absence) and fluorescence intensity as a relative measure of an antigen density.

CELL POPULATIONS IDENTIFIED BY FLOW CYTOMETRY

The surface and cytoplasmic markers expressed in hematologic malignancies resemble those of normal hematopoietic cell differentiation. Frequently, neoplastic cells are arrested at a particular stage of development and display aberrant antigenic patterns. Diagnosis and classification of hematologic neoplasms is based on the knowledge of normal hematopoietic maturation pathways.

In the past, the differentiation of hematopoietic cells was defined by morphologic criteria. Over time, it became clear that specific morphologic stages of development are accompanied by distinct changes in immunophenotype. Approximate morphologic-immunophenotypic correlates exist; however, because hematopoiesis is a continuous process, transitions between various developmental phases are not discrete.

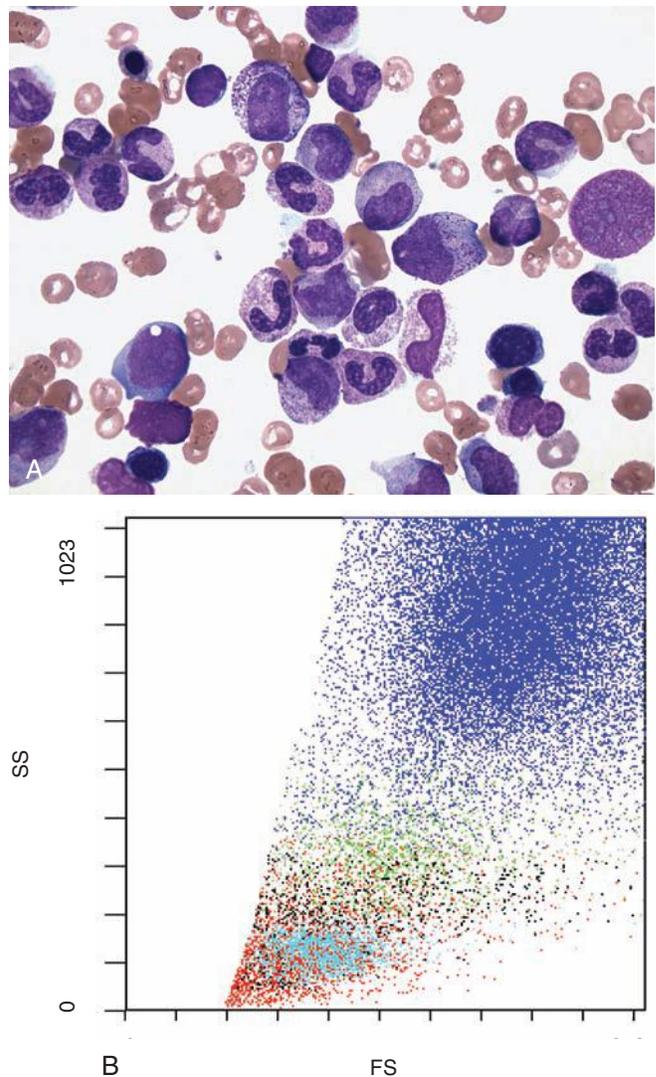


Figure 32-5 Main cell subpopulations of normal bone marrow. **A**, Bone marrow is composed of a heterogeneous population of cells of different sizes and variable complexity of cytoplasm (Wright-Giemsa stain, $\times 1000$). **B**, Dot plot of forward scatter (FS, cell size) versus side scatter (SS, internal complexity) reflects the heterogeneity of bone marrow subpopulations. Lymphocytes are smallest with negligible amount of agranular cytoplasm and are located closest to the origins of the axes (*aqua*). Monocytes are slightly larger with occasional granules and vacuoles (*green*). Granulocytic series shows prominent granularity (*navy*).

All hematopoietic progeny are derived from pluripotent stem cells. These cells are morphologically unrecognizable and are defined by their functional and antigenic characteristics. They usually express a combination of CD34, CD117 (*c-kit*), CD38, and HLA-DR antigens.⁹ As hematopoietic cells mature, they lose stem cell markers and acquire lineage-specific antigens. A brief discussion of the maturation sequence of major hematopoietic cell lineages is presented in the following sections.

Granulocytic Lineage

The stages of granulocytic lineage development, as defined by the expression of specific antigens, correspond closely to the morphologic sequence.¹⁰ The first morphologically recognizable cell committed to the granulocytic lineage is a

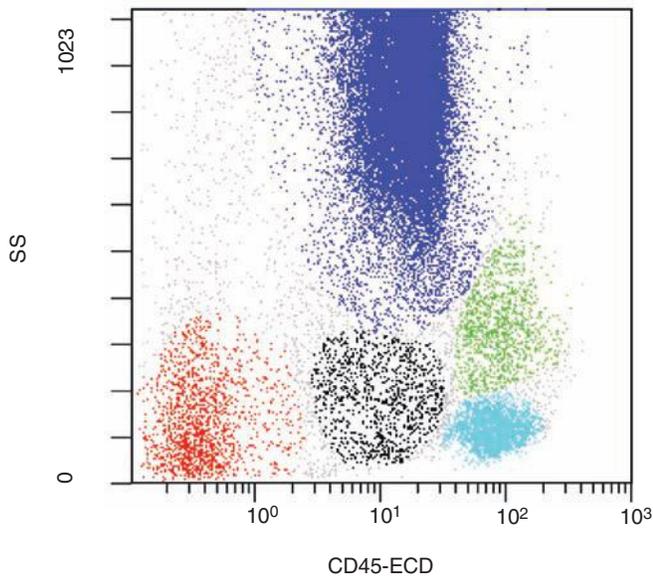


Figure 32-6 Scattergram showing differential densities of pan-hematopoietic marker CD45 on marrow leukocytes. Lymphocytes (*aqua*) and monocytes (*green*) show highest density of CD45 antigen. Intermediate expression of CD45 is seen in the granulocytic population (*navy*) and blasts (*black*). Erythroid precursors (*red*) are CD45⁻. ECD, phycoerythrin-Texas Red; SS, Side scatter.

myeloblast (Chapter 12). A myeloblast is characterized by an expression of immature cell markers CD34, CD38, HLA-DR, and stem cell factor receptor CD117. Pan-myeloid markers CD13 and CD33, present on all myeloid progeny, are first expressed at this stage. As a myeloblast matures to a promyelocyte, it loses CD34 and HLA-DR and acquires the CD15 antigen. Further maturation to a myelocyte stage leads to an expression of CD11b, a temporary loss of CD13, and a gradual decrease in the density of CD33. Finally, as granulocytic cells near a band stage, CD16 is acquired, and the density of CD13 increases.

Monocytic Lineage

The earliest immunophenotype stage of monocytic development is defined by a gradual increase in the density of CD13, CD33, and CD11b antigens. Subsequent acquisition of CD15 and CD14 marks the transition to a promonocyte and mature monocyte. In contrast to the granulocytic series, strong expression of CD64 and HLA-DR antigens persists throughout monocytic maturation.

Erythroid Lineage

The majority of erythroid precursors do not express pan-hematopoietic marker CD45. The earliest marker of erythroid differentiation is the transferrin receptor, CD71. The density of this antigen increases starting in the pronormoblast stage and is rapidly downregulated in reticulocytes.¹¹ In contrast, glycophorin A, although present on reticulocytes and erythrocytes, first appears at the basophilic normoblast stage.

Megakaryocytic Lineage

The maturation sequence of megakaryocytes is less well defined. CD41 and CD61, referred to as *glycoprotein IIb/IIIa complex*,

appear as the first markers of megakaryocytic differentiation. These antigens are present on a small subset of CD34⁺ cells believed to represent early megakaryoblasts.⁹ CD31 and CD36, although not entirely specific for megakaryocytic lineage, also are present on megakaryoblasts. Subsequent maturation to megakaryocytes and platelets is characterized by the appearance of additional glycoproteins, CD42, CD62P, and CD63.

Lymphoid Lineage

The B and T lymphocytes are derived from lymphoid progenitors that express CD34, terminal deoxynucleotidyl transferase (TdT), and HLA-DR. Lymphoid differentiation is characterized by a continuum of changes in the expression of surface and intracellular antigens. The earliest B cell markers include CD19, cytoplasmic CD22, and cytoplasmic CD79.¹² As B cell precursors mature, they acquire the CD10 antigen. The appearance of the mature B cell marker CD20 coincides with the decrease in CD10 antigen expression. Another specific immature B cell marker is the cytoplasmic μ chain that eventually is transported to the surface and forms the B cell receptor. At this stage, the immunoglobulin chains in so-called naive B cells have become rearranged. The normal mature B cell population shows a mix of κ and λ light chain-expressing cells. The exclusive expression of only κ or λ molecules is a marker of monoclonality, seen frequently in mature B cell neoplasms. The differentiation of mature naive B cells, often recapitulated by B cell malignancies, is discussed in detail in Chapter 36.

Similar to B cell precursors, immature T cells express CD34 and TdT.¹³ The first markers associated with T cell lineage include CD2, CD7, and cytoplasmic CD3. CD2 and CD7 are also present in natural killer (NK) cells and, along with the CD56 molecule, are used to detect NK cell-derived neoplasms. In T cells, the expression of CD2, CD7, and cytoplasmic CD3 is followed by the appearance of CD1a and CD5 and coexpression of CD4 and CD8 antigens. Finally, the CD3 antigen appears on the cell surface, and CD4 or CD8 is lost. The sequential transition from double-negative (CD4⁻CD8⁻) through double-positive (CD4⁺CD8⁺) stages generates a population of mature helper (CD4⁺) and suppressor (CD8⁺) T cells. T cell differentiation occurs in the thymus.

FLOW CYTOMETRIC ANALYSIS OF MYELOID NEOPLASMS (ACUTE MYELOID LEUKEMIAS AND CHRONIC MYELOID NEOPLASMS)

In myeloid malignancies, flow cytometry is used for initial diagnosis, follow-up, and prognostication. Specific immunophenotypes are associated with select cytogenetic abnormalities. Because most myeloid malignancies are stem cell disorders, the evaluation of blast population and maturing myeloid component is considered mandatory. Almost invariably, blasts are characterized by a low-density expression of CD45 antigen. In normal bone marrow, a blast gate includes a relatively low number of cells showing the immature myeloid immunophenotype (Figure 32-6). In acute myeloid and lymphoblastic leukemias, this region becomes densely populated by immature cells, which reflects the increased number of blasts seen in the bone marrow (Figure 32-7). The exact position of the immature population on the CD45/SS

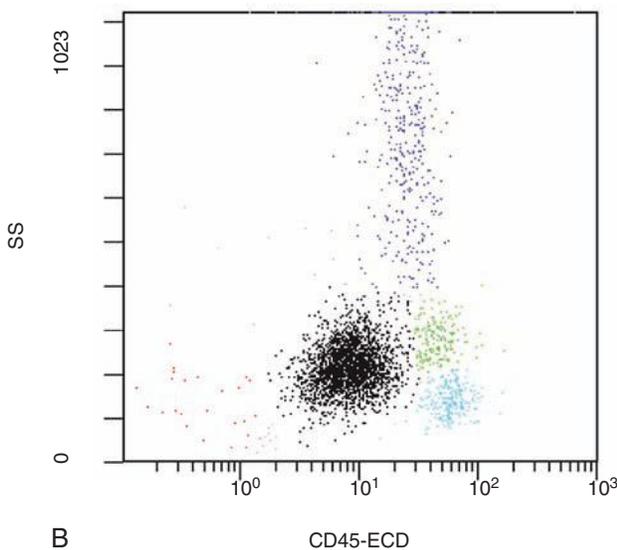
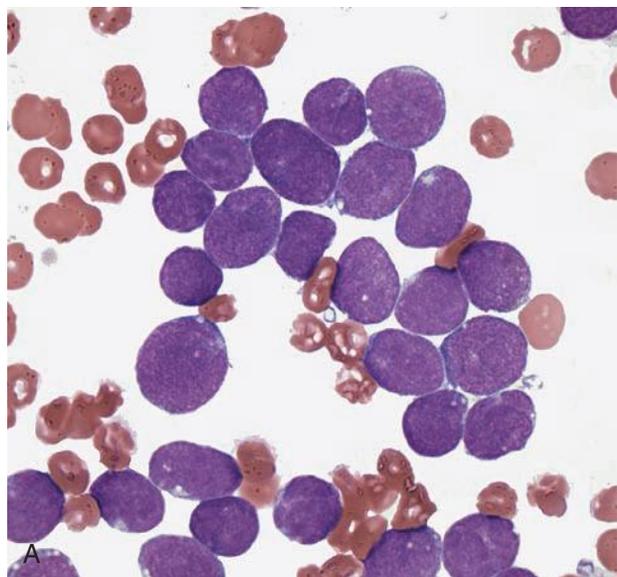


Figure 32-7 Bone marrow specimen showing acute leukemia. Note uniform cytologic and flow cytometric characteristics. **A**, Bone marrow aspirate from a patient with acute lymphoblastic anemia (Wright-Giemsa stain, $\times 500$). **B**, CD45 versus side scatter (SS) plot shows a homogeneous population of blasts with a marked decrease in normal hematopoietic elements. Compare with the heterogeneous pattern of normal bone marrow in Figure 33-6. *ECD*, Phycoerythrin-Texas Red.

displays depends on the subtype of acute myeloid leukemia (AML). In this chapter, the immunophenotypic features of AML and chronic myeloid neoplasms are discussed in the context of the World Health Organization classification, which introduced categories defined by recurrent cytogenetic abnormalities.¹ These leukemias often show specific immunophenotypes and are discussed separately in the following sections.

Acute Myeloid Leukemias with Recurrent Cytogenetic Abnormalities

In most cases, AML with $t(8;21)(q22;q22);RUNX1/RUNX1T1$ shows an immature myeloid immunophenotype with high-density CD34 and coexpression of CD19 (Figure 32-8).¹⁴ In

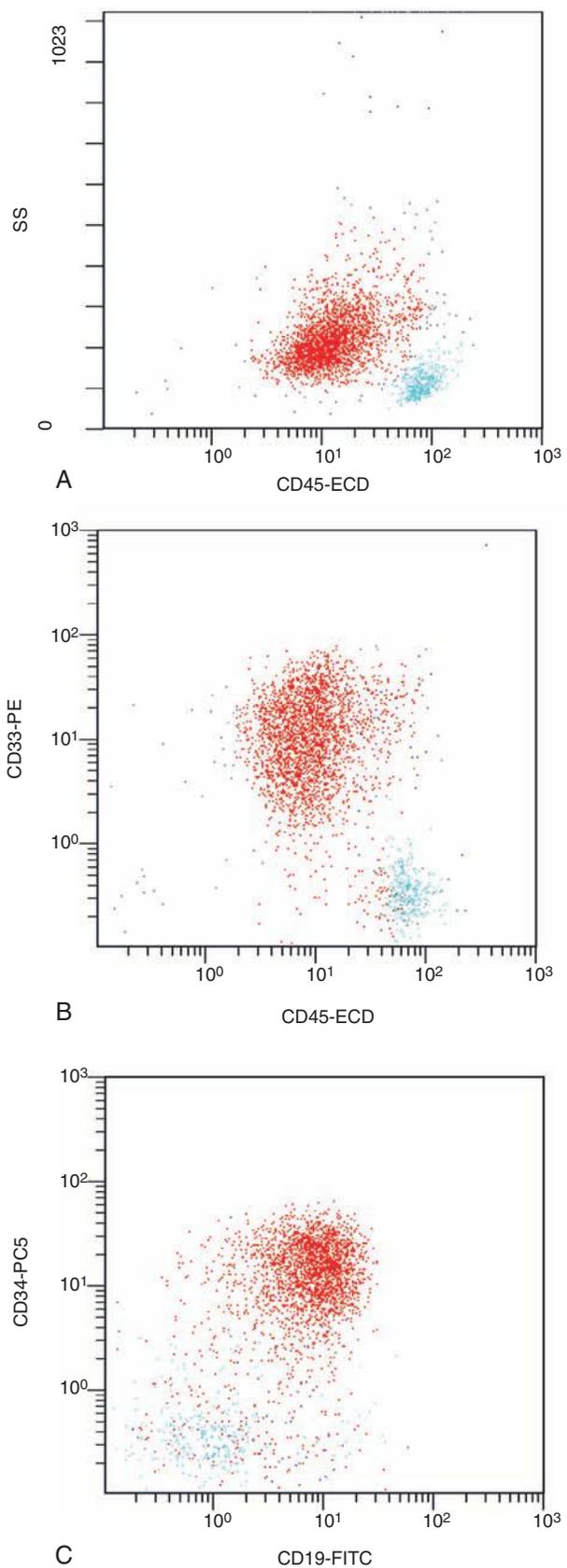


Figure 32-8 Acute myeloid leukemia with $t(8;21)(q22;q22);RUNX1-RUNX1T1$. **A**, CD45 versus side scatter (SS) showing increase in blasts (red) with residual lymphocytes (aqua). **B** and **C**, Blasts are positive for CD33 and CD34 with characteristic coexpression of CD19 antigen. *ECD*, Phycoerythrin-Texas Red; *FITC*, fluorescein isothiocyanate.

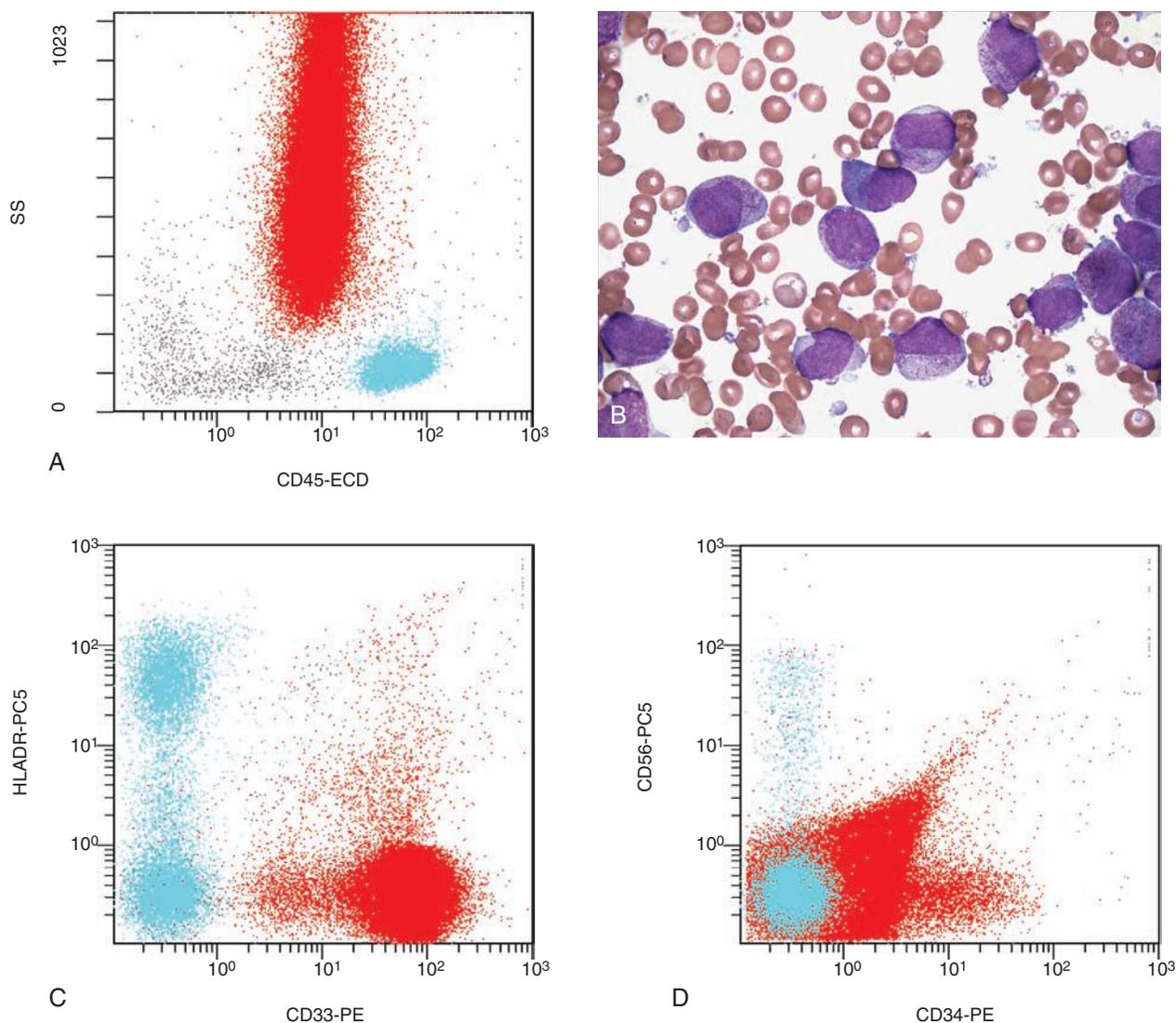


Figure 32-9 Acute promyelocytic leukemia. **A**, Typical side scatter (SS) pattern in acute promyelocytic leukemia corresponding to prominent granularity of leukemic cells (red). Residual lymphocytes are shown in aqua. **B**, Numerous leukemic promyelocytes with distinct granules and occasional Auer rods (Wright-Giemsa stain, $\times 1000$). **C**, Leukemic cells show high-density expression of CD33 antigen and lack HLA-DR. **D**, Similarly, CD34 antigen is absent or present in only a few leukemic cells. *ECD*, phycoerythrin-Texas Red; *PC5*, phycoerythrin-cyanine 5; *PE*, phycoerythrin; *SS*, Side scatter.

addition, numerous myeloid antigens, including CD33, CD13 and myeloperoxidase, are expressed. Frequently, there is asynchronous coexpression of CD34 and CD15. TdT is commonly present.

AML with $\text{inv}(16)(p13.1q22)$ or $\text{t}(16;16)(p13.1;q22)$; *CBFB/MYH11* is characterized by the presence of immature cells with expression of CD34, CD117, and TdT and a subpopulation of maturing cells showing monocytic (CD14, CD11b, CD4) and granulocytic (CD15) markers.¹⁵ The aberrant coexpression of CD2 on the monocytic population is common.

Acute promyelocytic leukemia, AML with $\text{t}(15;17)(q22;q12)$; *PML/RARA*, shows a specific immunophenotype. In contrast to most less-differentiated myeloid leukemias, acute promyelocytic leukemia manifests with high SS, which reflects the granular cytoplasm of leukemic cells (Figure 32-9). The constellation of immunophenotypic features used to diagnose acute promyelocytic leukemia includes lack of CD34 and HLA-DR antigens, presence of homogeneous strong CD33 along with myeloperoxidase, and variable CD13 and CD15.¹⁶

AMLs with $\text{t}(9;11)(p22;q23)$; *MLLT3/MLL* in adults most commonly present with monocytic differentiation. The immunophenotypic features are nonspecific and can be seen in any acute myelomonocytic or monocytic leukemia (negative for CD34 and positive for CD33, CD13, CD14, CD4, CD11b, and CD64).

Acute Myeloid Leukemias Not Otherwise Specified

In the least-differentiated AMLs—AML with minimal differentiation and AML without maturation—blasts are present in the region of low-density CD45 antigen and display low SS reflecting their relatively agranular cytoplasm. Even the least differentiated AML with minimal differentiation is usually positive for myeloid markers. The expression of CD13, CD33, and CD117 is common. Primitive hematopoietic antigens such as CD34 and HLA-DR are often seen. Myeloperoxidase is absent or is expressed in only a few cells. The immunophenotypic profile of AML with maturation is similar, but more

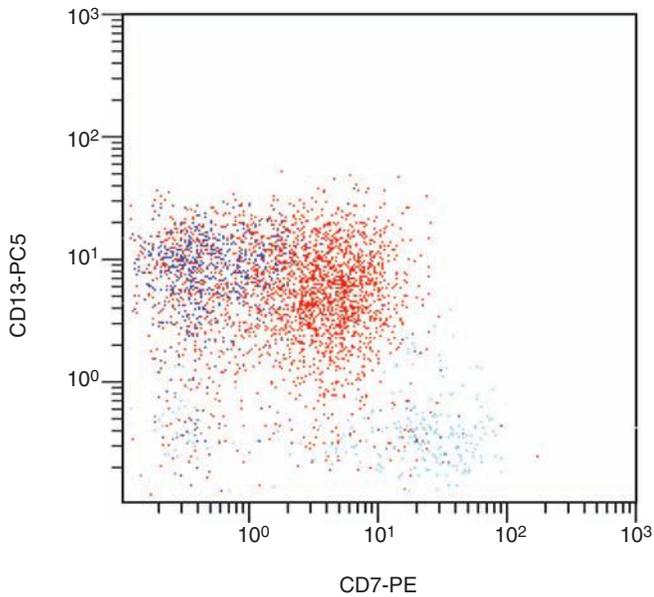


Figure 32-10 Myeloblasts of acute myeloid leukemia show aberrant co-expression of CD7 antigen. *PE*, Phycoerythrin; *PC5*, phycoerythrin-cyanine 5.

mature myeloid markers such as CD15 and myeloperoxidase are often expressed.

Occasionally, there is aberrant coexpression of antigens. Simultaneous expression of early and late markers of myeloid differentiation on the leukemic blasts is not uncommon (asynchronous antigen expression). Similarly, markers specific for other lineages, such as lymphoid lineage may be seen on myeloid blasts. The most common example is CD7 antigen, which is usually present in the T/NK cell population (Figure 32-10).

Acute myelomonocytic leukemia and acute monoblastic leukemia usually show higher expression of CD45, similar to normal monocytic precursors. In addition, in acute myelomonocytic leukemia, a population of primitive myeloid blasts is often seen (Figure 32-11). The expression of myeloid markers and antigens associated with monocytic lineage, such as CD14, CD4, CD11b, and CD64, is commonly seen. Although CD14 is present on all mature monocytes, it may be absent in monocytic leukemias.¹⁷ More immature monocytic markers, such as CD64, are more consistently expressed.

Acute erythroid leukemias are categorized into two subtypes: pure erythroid leukemia and erythroleukemia (erythroid/myeloid

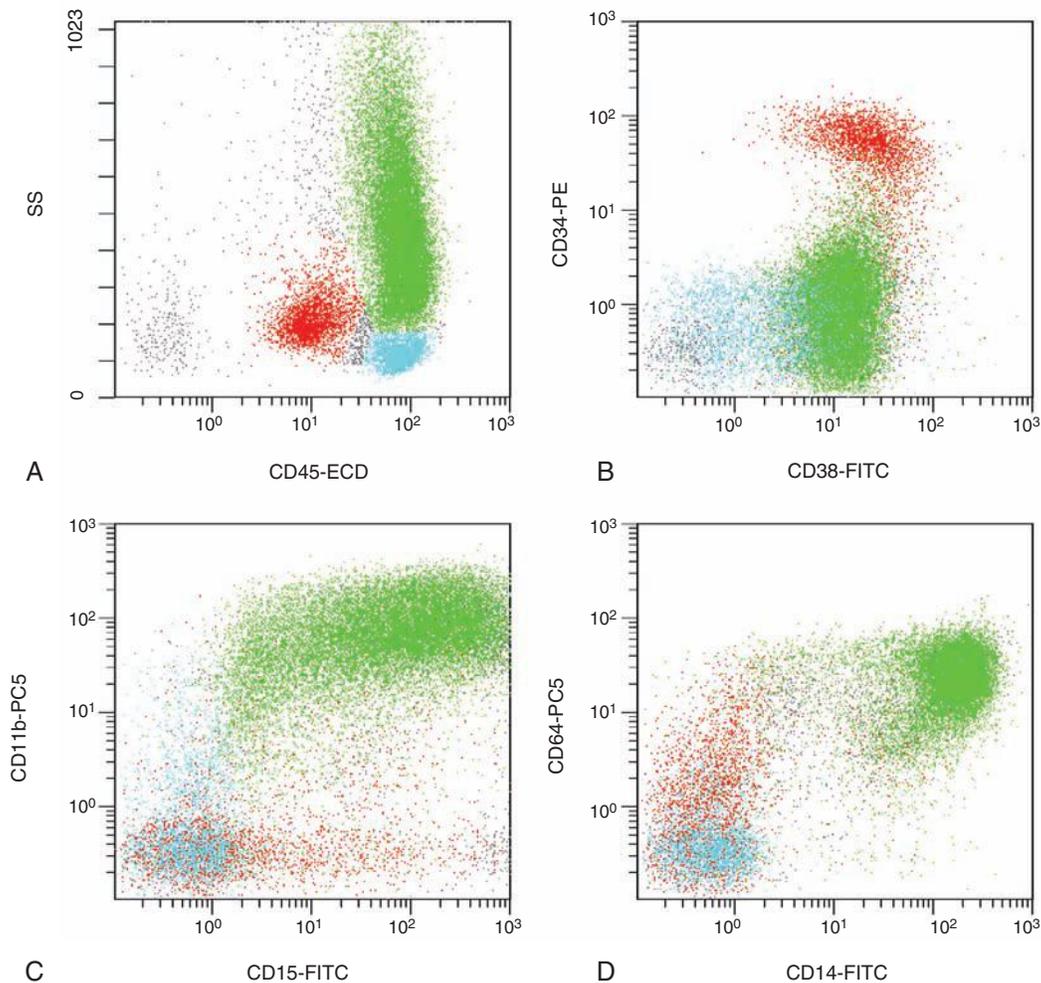


Figure 32-11 Peripheral blood immunophenotyping in acute myelomonocytic leukemia. **A**, CD45 versus side scatter (SS) display shows myeloid blasts (*red*) and a monocytic population (*green*). **B** through **D**, Primitive leukemic blasts are positive for CD34 and negative for CD14. In contrast, monocytic population does not express CD34 and shows positivity for mature monocyte marker CD14 and characteristic monocytic pattern of CD11b and CD15 expression. *ECD*, Phycoerythrin-Texas Red; *FITC*, fluorescein isothiocyanate; *PC5*, phycoerythrin-cyanine 5.

leukemia). In the latter, primitive myeloid blasts and erythroid precursors are present. Leukemic cells are positive for erythroid markers such as CD71, glycophorin A, and hemoglobin (HGB). In more immature erythroid leukemias, glycophorin A and hemoglobin may be absent. In these cases, the diagnosis is based on the absence of myeloid markers, high expression of CD71, and scatter characteristics.

Acute megakaryoblastic leukemia usually shows low SS and low to absent CD45. Early megakaryocytic markers, CD41 and CD61, are frequently expressed.¹⁸ Occasionally, the late megakaryocytic marker CD42 is present. The expression of stem cell markers CD34 and HLA-DR on the population of leukemic megakaryoblasts varies.

Myeloproliferative Neoplasms and Myelodysplastic Syndromes

The knowledge of antigen expression in the normal differentiation of myeloid lineages allows us to define the aberrant expression patterns frequently seen in chronic myeloid disorders. The abnormalities detected by flow cytometry reflect morphologic features (e.g., hypogranulation of neutrophils in myelodysplastic syndrome detected by low SS) and show changes in antigen expression. Qualitative (presence or absence of a particular antigen) and quantitative abnormalities (differences in the number of antigen molecules) can be used for diagnostic purposes. The interested reader is referred to review articles discussing the details of immunophenotyping in myelodysplastic syndromes and myeloproliferative neoplasms.¹⁹⁻²¹ A few examples are highlighted to illustrate the role of flow cytometry in diagnosing these diseases.

SS abnormalities related to hypogranulated neutrophils are seen in approximately 70% of myelodysplastic syndromes (Figure 32-12). In high-grade myelodysplastic syndrome and myeloproliferative neoplasms undergoing transformation, the increase in immature cells is detected easily. Blasts have a variety of aberrant immunophenotypic features, most commonly coexpression of CD7 and CD56 antigens. Blasts and maturing granulocytic precursors may show asynchronous expression of myeloid markers, including retention of CD34 and HLA-DR in late stages of maturation or late myeloid markers presenting early in differentiation, such as CD15 on myeloblasts. Asynchronous coexpression of markers can also be seen in monocytic and erythroid lineages. Aberrant immunophenotypes are seen in 98% of cases of myelodysplastic syndrome. More importantly, immunophenotypic abnormalities can be seen in cases with minimal or no morphologic dysplasia.¹⁹ Other studies underscore the significance of immunophenotypic abnormalities in predicting the outcome after stem cell transplantation.^{22,23}

The utility of flow cytometry in myeloproliferative neoplasms is less well established. Specifically, the application of flow cytometry as a diagnostic tool in chronic myelogenous leukemia is limited to the accelerated or blast phase, in which a lineage of an expanding blast population needs to be determined. In the chronic phase, the presence of *BCR/ABL1* rearrangement (Philadelphia chromosome) demonstrated by conventional karyotyping or molecular studies remains the defining feature of the disease. Other myeloproliferative neoplasms are not well studied. In general, flow cytometric

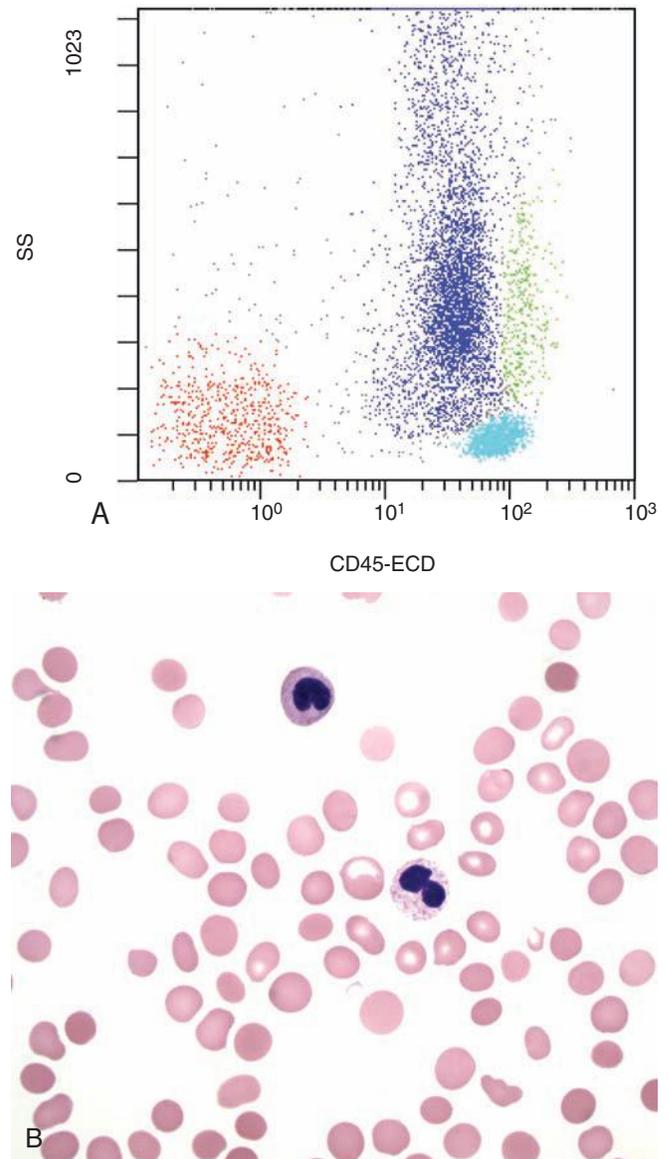


Figure 32-12 A, Low side scatter (SS) of hypogranular neutrophils seen in most cases of myelodysplastic syndrome (*nav*). B, Corresponding photomicrograph of markedly dysplastic, hypogranulated neutrophils in myelodysplastic syndrome (Wright-Giemsa stain, $\times 1000$). ECD, Phycoerythrin-Texas Red.

abnormalities are seen in most cases with abnormal karyotype.²⁰ No consistent set of immunophenotypic features that can be routinely used in the workup of myeloproliferative states has been described.

FLOW CYTOMETRIC ANALYSIS OF LYMPHOID NEOPLASMS (LYMPHOBLASTIC LEUKEMIA/LYMPHOMA AND MATURE LYMPHOID NEOPLASMS)

Similar to myeloid neoplasms, a diagnosis of lymphoid malignancies relies on the expression of lineage-associated markers corresponding to specific stages of lymphoid development. No single marker can be used for lineage assignment, and a diagnosis is typically based on the presence of several B cell or T cell antigens. The sentinel feature of mature B and T cells is the

presence of surface receptor complexes. The immune system responds to a wide array of antigens; in healthy individuals, B and T cells express a great diversity of surface immunoglobulin and T cell receptor complexes (polyclonal populations). A neoplastic lymphoid population is characterized by the monoclonal expression of a single B or T cell receptor. In most cases, clonality confirms the malignant nature of lymphoid proliferation. In contrast, lymphoid precursors are generally negative for surface immunoglobulin and T cell receptors and instead carry immature markers. In lymphoblastic (precursor-derived) neoplasms, an expansion of a population with homogeneous marker expression, rather than clonality, is diagnostic of malignancy. The following section presents the key immunophenotypic features of lymphoblastic leukemias and lymphomas. Selected examples of the association between the immunophenotype and the genotype are discussed.

B Lymphoblastic Leukemia/Lymphoma

B lymphoblastic leukemia/lymphoma (B-LL) is also referred to as B acute lymphoblastic leukemia or B lymphoblastic

lymphoma. B lymphoblasts are positive for CD19, CD22, CD79a, HLA-DR, and TdT (Figure 32-13). The expression of CD34 and CD10 is frequently seen. Surface immunoglobulin light chains are not present. Cytoplasmic μ chain or surface immunoglobulin M may be detected, however. Because B-LL can arise at any stage of B cell differentiation, the presence of several specific markers usually defines early precursor, intermediate, and pre-B stages. Frequently, immunophenotypes correlate with specific cytogenetic and clinical features. In routine practice, confirmation of cytogenetic abnormality using conventional karyotyping or molecular techniques is necessary.

B Lymphoblastic Leukemia/Lymphoma with *t(v;11q23);MLL Rearranged*

MLL gene rearrangements occur most frequently in infant B-LL. Unlike in most B-LLs, blasts in this leukemia are negative for CD10 antigen.²⁴ CD19, CD34, TdT, and occasional myeloid markers are present. The more mature B cell marker CD20 is absent.

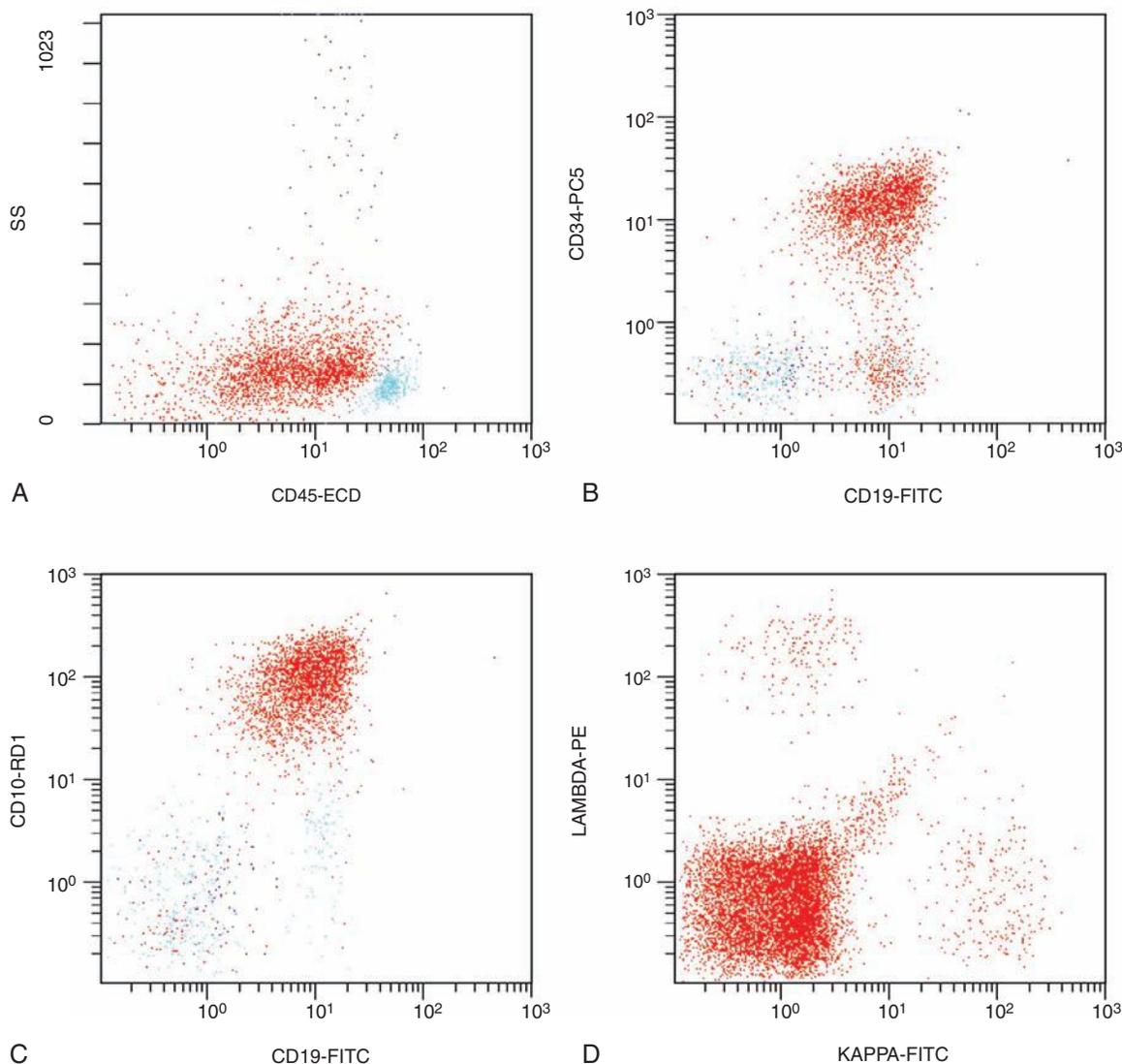


Figure 32-13 B lymphoblastic leukemia/lymphoma. **A**, Low-density CD45 antigen characteristic of the blast population. **B**, Uniform expression of CD34 and CD19 on leukemic blasts. **C**, High-density CD10 on CD19+ blasts. **D**, Lack of surface κ and λ light chains signifies immature B cell population. *ECD*, phycoerythrin-Texas Red; *FITC*, fluorescein isothiocyanate; *PC5*, phycoerythrin-cyanine 5; *PE*, phycoerythrin; *SS*, Side scatter.

B Lymphoblastic Leukemia/Lymphoma with t(9;22) (q34;q11.2);BCR/ABL1

Philadelphia chromosome, t(9;22);BCR/ABL1, is a hallmark of chronic myelogenous leukemia but also can occur in pediatric and adult B-LL. These cases benefit from an addition of tyrosine kinase inhibitor to the chemotherapy regimen, so it is important to identify them promptly. Most BCR/ABL1-positive cases have a classic intermediate or common B-LL immunophenotype with the expression of CD19, CD10, CD34, and TdT. The expression of myeloid markers CD13 and CD33 and the lack or decreased expression of CD38 antigen on leukemic blasts are common. The density of antigens and their homogeneous or heterogeneous expression within leukemic populations correlates closely with the presence of BCR-ABL1.²⁵

T Lymphoblastic Leukemia/Lymphoma

T lymphoblastic leukemia/lymphoma (T-LL) is derived from immature cells committed to T cell lineage. Designation of leukemia or lymphoma depends on the primary site of involvement:

bone marrow or lymph node. T-LL expresses a combination of markers reflecting the stage of T cell differentiation. CD3 is the most specific T cell marker. As with normal T cells, this antigen is seen initially in the cytoplasm before appearing on the cell surface. Other T cell antigens include CD2, CD7, CD5, CD1a, CD4, and CD8. Usually a series of these antigens is detected, recapitulating the T cell differentiation (Figure 32-14). CD34 and CD10 may be present. As in other lymphoid neoplasms, the panel of markers determines the lineage.

Mature Lymphoid Neoplasms

B and T cell lymphomas display immunophenotypes resembling their normal counterparts. The immunophenotypic features of lymphomas are discussed in detail in Chapter 36 and are summarized in Table 36-2. The flow cytometric workup of lymphomas is facilitated by the clonal origin of mature lymphoid neoplasms, which implies that the malignant population is derived from a single cell. Therefore, all neoplastic cells typically show similar genetic and immunophenotypic features. This stands in strong

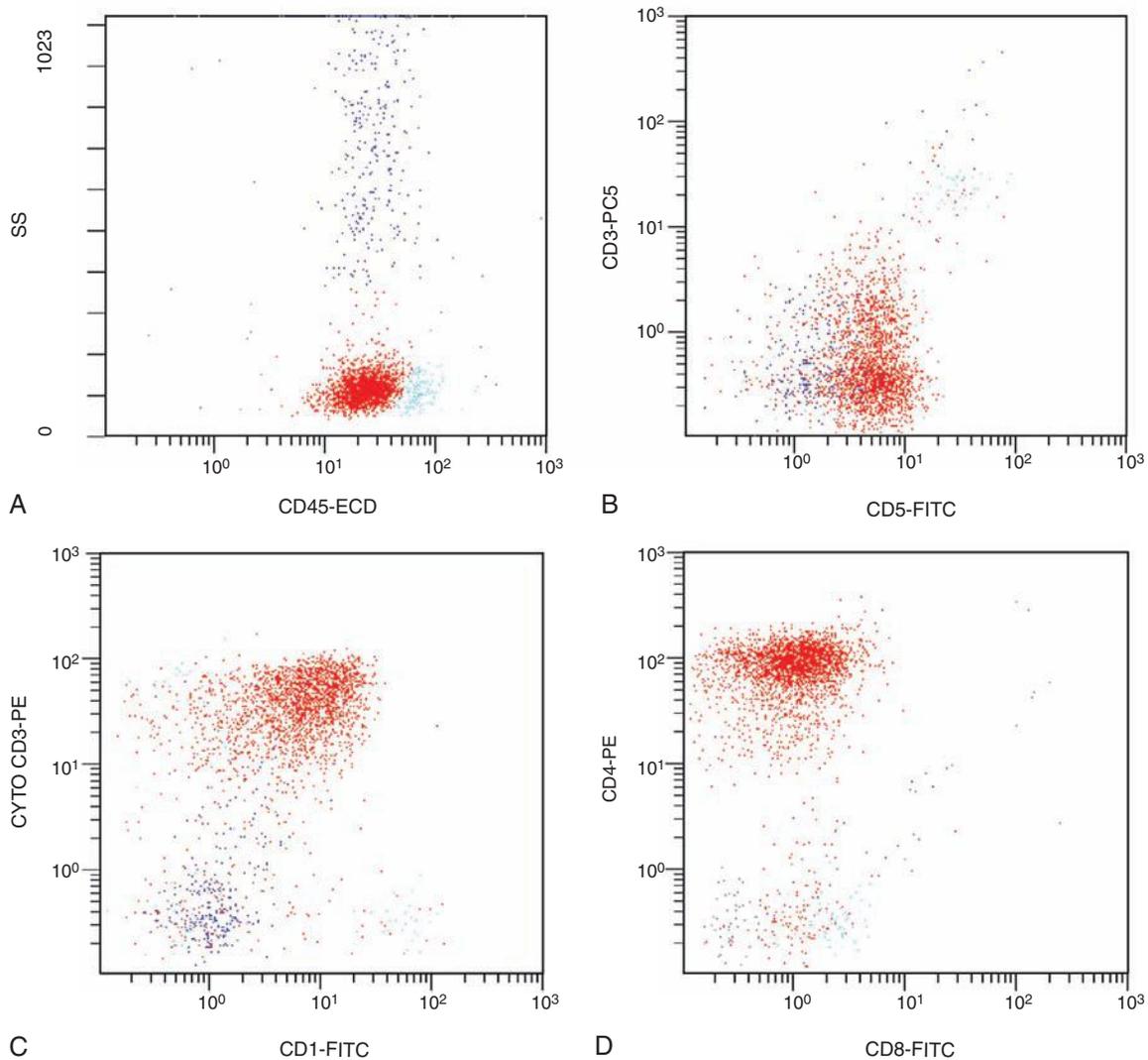


Figure 32-14 T lymphoblastic leukemia/lymphoma. **A**, Predominant population in the blast gate. **B** and **C**, Although CD3 antigen is absent from the surface of leukemic cells, it is present in blast cytoplasm, confirming the precursor T cell origin of the leukemia (**C**). Note residual normal T cells (*aqua*) positive for surface CD3 and CD5 antigens. **D**, Simultaneous expression of CD4 and CD8 antigens. ECD, phycoerythrin-Texas Red; FITC, fluorescein isothiocyanate; PC5, phycoerythrin-cyanine 5; PE, phycoerythrin; SS, Side scatter.

contrast to variable immunophenotypes of normal lymphoid populations, reflecting a process of antigen-driven selection.

Mature B Cell Neoplasms

Normal precursor B cells randomly rearrange immunoglobulin heavy and light chain genes. As a result, a mature B cell population expresses a mix of heavy and light chains (Figure 32-15, A). In contrast, a monoclonal surface light chain expression, exclusively κ or λ , is seen in most B cell lymphomas (Figure 32-15, B). Light chain monoclonality along with the expression of pan-B cell markers is diagnostic of B cell lymphoma. Rarely, lymphomas may lose the expression of surface light chains, a feature not seen in normal mature B cells.²⁶ In most cases of plasma cell myeloma, neoplastic plasma cells lack surface immunoglobulin light chains and express only cytoplasmic κ or λ .

Mature T Cell Neoplasms

In T cells, similar to B cells, clonality in most cases indicates malignancy. In the past, the clonality of T cells could only be confirmed by using a molecular analysis of T cell receptor genes. Recently, a flow cytometric assay has been shown to detect clonality in most cases of T cell lymphoma.²⁷ This technique uses a broad array of antibodies against variable regions of T cell receptors. Because this methodology is not widely available, often a diagnosis of T cell lymphoma is based on aberrant immunophenotype. In most cases, a loss or atypical expression of a lymphoid marker can be shown using flow cytometry. For example, mycosis fungoides/Sézary syndrome is characterized by a mature T cell immunophenotype with expression of CD2, surface CD3, CD5, and CD4 and with a loss of the CD7 antigen (Figure 32-16). Over the

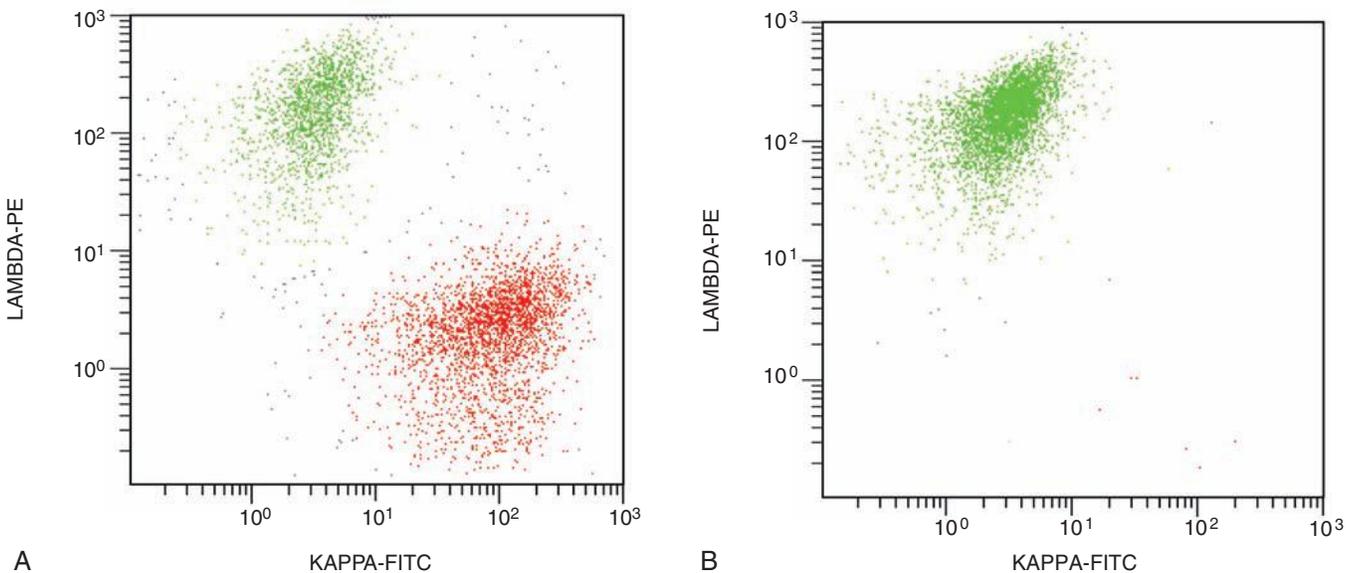


Figure 32-15 Comparison of surface light chain expression in reactive and malignant B cells. **A**, Reactive B cells show heterogeneous expression of κ and λ . **B**, B cell lymphomas are monoclonal, with the entire lymphoma population expressing only one type of light chain. FITC, Fluorescein isothiocyanate; PE, phycoerythrin.

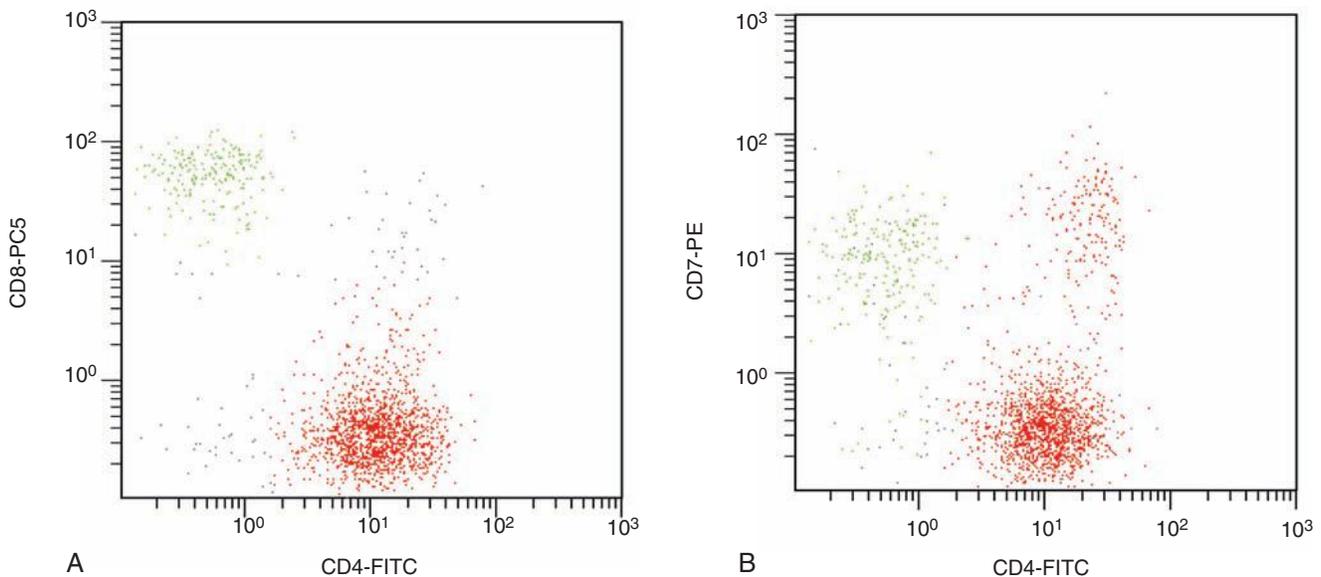


Figure 32-16 Mycosis fungoides. **A**, T cell population is positive for CD4 antigen (red). **B**, Neoplastic T cells show loss of CD7. Red represents CD4 and the expression of CD7 (green) is very low. FITC, Fluorescein isothiocyanate; PE, phycoerythrin; PC5, phycoerythrin-cyanine 5.

years it has been shown that the aberrant immunophenotype is a reliable diagnostic feature when the neoplastic population is sizeable. However, small numbers of T cells with unusual antigen makeup can appear in inflammatory conditions;²⁸ thus the aberrant immunophenotype alone cannot be considered pathognomonic of T cell malignancy.

OTHER APPLICATIONS OF FLOW CYTOMETRY BEYOND IMMUNOPHENOTYPING OF HEMATOLOGIC MALIGNANCIES

The immunophenotyping of hematolymphoid neoplasms is one of many applications of flow cytometry. Other common applications include a diagnosis and monitoring of immunodeficiency states, diagnosis of paroxysmal nocturnal hemoglobinuria (PNH), stem cell enumeration, cell cycle analysis, detection of fetal hemoglobin, and monitoring of sepsis.

Select primary (inherited) and secondary (acquired) immunodeficiencies can be diagnosed using flow cytometry. Both a loss of specific antigens (e.g., CD11/CD18 in leukocyte adhesion deficiency) and functional defects (e.g., oxidative burst evaluation in chronic granulomatous disease) can be assayed by flow cytometry.

Human immunodeficiency virus infection causes a progressive decrease in the number of CD4⁺ helper T cells. The absolute number of helper T cells in peripheral blood correlates with the stage of the disease and with patient prognosis. The enumeration of T cells and their subsets is easily accomplished by flow cytometry using antibodies against CD4 and CD8 antigens. The absolute numbers are derived by performing a routine white blood cell (WBC) count on the concurrent peripheral blood specimen or by running calibrating beads simultaneously with the patient sample. The CD4:CD8 ratio in healthy individuals is typically greater than 1. There is a significant decrease in numbers of CD4 positive T cells in HIV-positive patients resulting in a reversed CD4:CD8 ratio. Since the CD4 lymphocyte depletion is associated with various infections, the absolute number of CD4 positive lymphocytes serves also as a guide for antibiotic prophylaxis in HIV positive patients.

The diagnostic approach to PNH is a prime example of how an application of flow cytometry increases understanding of hematologic disorders and directly contributes to clinical decision making (Chapter 24).²⁹ Before the development of the flow cytometric assay, PNH diagnosis was based on detection of increased susceptibility of RBCs to lysis by the Ham or sucrose hemolysis tests, both of which showed inconsistent sensitivity. Flow cytometry significantly improved the sensitivity and specificity of PNH testing. The absence or decreased expression of glycosylphosphatidylinositol-anchored proteins on RBCs, granulocytes, and monocytes as measured by flow cytometry is diagnostic of PNH. In addition, the levels of CD59 expression correlate with clinical symptoms (Figure 32-17).

Another important application of flow cytometry is cell sorting. During sorting, a heterogeneous cell population is physically divided into subsets according to their physical or

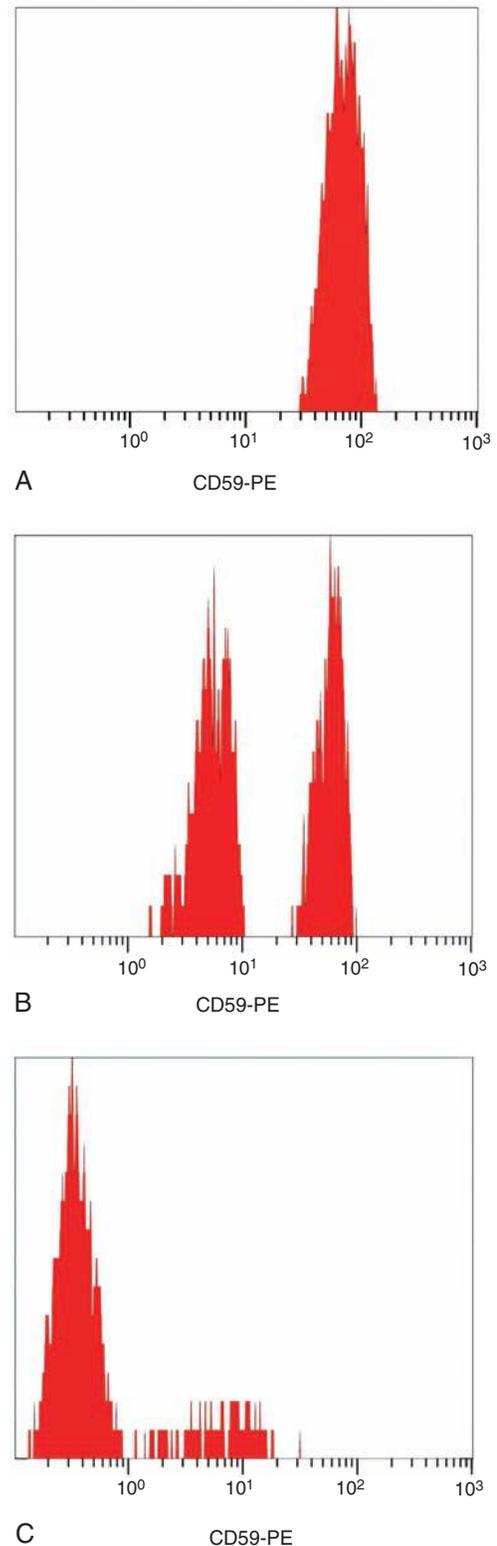


Figure 32-17 Diagnosis of paroxysmal nocturnal hemoglobinuria (PNH) is based on the decreased expression of glycosylphosphatidylinositol (GPI)-linked molecules. Different levels of GPI-anchored proteins are best visualized in red blood cells (RBCs) using an antibody against CD59 antigen. **A**, RBCs from a healthy volunteer show a high number of CD59 molecules and correspond with type I cells. **B**, Varying percentages of type I cells (normal level of CD59 antigen) and a population with slight decrease in CD59 expression (type II cells) can be seen in PNH patients. **C**, Granulocytes with a complete loss of CD59 (type III cells) in a patient with PNH. This patient received numerous red blood cell transfusions; the loss of CD59 is best shown in granulocytic and monocytic populations. PE, Phycoerythrin.

immunophenotypic properties. High-speed sorting is achieved by charging droplets containing individual cells of interest. As the charged droplet passes through the electrostatic field, it is isolated from the remainder of the sample and collected into a separate container. The primary clinical application of cell sorting is in stem cell transplantation.

For years, flow cytometry remained confined to the hematopathology and research laboratories. Currently, this methodology is used in bone marrow transplantation, transfusion medicine, coagulation, microbiology, molecular pathology, and drug development. Specific examples of novel applications include tissue typing, molecular testing for neoplasia-

associated translocations, and follow-up of drug response, such as by monitoring platelet activation after antiplatelet therapy.

Flow cytometry is a mature field that in recent years experienced a revival with a focus on high-throughput testing for simultaneous analysis of multiple biologic constituents. New approaches to a single-cell analysis such as spectral flow cytometry and an integration of mass spectrometry with single-cell fluidics provide a superior resolution and expand the number of parameters that can be measured in any given cell. These methodologies are in development and open new avenues to diagnostic immunophenotyping in hematopathology.^{30,31}

SUMMARY

- Flow cytometry measures physical, antigenic, and functional properties of particles suspended in a fluid.
- Multiparameter flow cytometry is a technique routinely used for a diagnosis and follow-up of hematologic disorders.
- The characterization of complex specimens is achieved through the analysis of individual cells for multiple parameters and the simultaneous display of data for thousands of cells. The cell size, cytoplasmic complexity, and immunophenotypic features detected by monoclonal antibodies directly conjugated to various fluorochromes are analyzed in clinical specimens.
- A key starting point in flow cytometric analysis is a high-quality fresh specimen.
- A flow cytometer consists of fluidics, a light source (laser), multiple detectors, and a computer.
- As with microscopic examination, an evaluation of flow cytometric data is based on the inspection of visual patterns. Initially, the entire sample is scanned for the presence of abnormal populations. Subsequently, detailed immunophenotypic features of cell subsets are studied.
- The immunophenotyping of hematologic specimens is based on knowledge of the maturation patterns of hematopoietic cells. In comparison, myeloid and lymphoid malignant cells and cell populations in nonneoplastic hematologic disorders show significant qualitative and quantitative differences in antigen expression.
- Flow cytometric analysis of acute leukemia determines a lineage of leukemic cells. In select entities, immunophenotype corresponds to the underlying genetic lesion.
- Immunophenotyping of myelodysplastic syndromes and chronic myeloproliferative neoplasms is an emerging application of clinical flow cytometry.
- The clonality of mature B cell and T cell neoplasms can be detected by flow cytometry.
- Flow cytometric analysis is used for diagnosis and monitoring of immunodeficiencies, stem cell enumeration, detection of fetal hemoglobin, tissue typing, molecular analysis, and drug testing.

Now that you have completed this chapter, go back and read again the case studies at the beginning and respond to the questions presented.

REVIEW QUESTIONS

Answers can be found in the Appendix.

1. What is the most common clinical application of flow cytometry?
 - a. Diagnosis of platelet disorders
 - b. Detection of fetomaternal hemorrhage
 - c. Diagnosis of leukemias and lymphomas
 - d. Differentiation of anemias
2. Which of the following is true of CD45 antigen?
 - a. It is present on every cell subpopulation in the bone marrow.
 - b. It is expressed on all hematopoietic cells, with the exception of megakaryocytes and late erythroid precursors.
 - c. It is not measured routinely in flow cytometry.
 - d. It may be present on nonhematopoietic cells.
3. Erythroid precursors are characterized by the expression of:
 - a. CD71
 - b. CD20
 - c. CD61
 - d. CD3
4. In [Figure 32-2A](#), the cell population colored in aqua represents:
 - a. Monocytes
 - b. Nonhematopoietic cells
 - c. Granulocytes
 - d. Lymphocytes

5. Antigens expressed by B-LL include:
 - a. CD3, CD4, and CD8
 - b. CD19, CD34, and CD10
 - c. There are no antigens specific for B-LL.
 - d. Myeloperoxidase
6. Which of the following is true of flow cytometric gating?
 - a. It is best defined as selection of a target population for flow cytometric analysis.
 - b. It can be done only at the time of data acquisition.
 - c. It can be done only at the time of final analysis and interpretation of flow cytometric data.
 - d. It is accomplished by adjusting flow rate.
7. Collection of ungated events:
 - a. Facilitates comprehensive analysis of all cells
 - b. Does not help in detection of unexpected abnormal populations
 - c. Allows the collection of data on a large number of rare cells
 - d. Is used for leukemia diagnosis only
8. Mycosis fungoides is characterized by:
 - a. Loss of certain antigens compared with the normal T cell population
 - b. Polyclonal T cell receptor
 - c. Immunophenotype indistinguishable from that of normal T cells
 - d. Expression of CD3 and CD8 antigens
9. Mature granulocytes show the expression of:
 - a. CD15, CD33, and CD34
 - b. CD15, CD33, and CD41
 - c. CD15, CD33, and CD13
 - d. CD15, CD33, and CD7
10. During the initial evaluation of flow cytometric data, cell size, cytoplasmic complexity, and expression of CD45 antigen are used to define cell subpopulations. Which of the following parameters defines cytoplasmic complexity/granularity?
 - a. SS
 - b. FS
 - c. CD45
 - d. HLA-DR
11. The most important feature of the mature neoplastic B cell population is:
 - a. The presence of a specific immunophenotype with expression of CD19 antigen
 - b. A clonal light chain expression (i.e., exclusively κ - or λ -positive population)
 - c. A clonal T cell receptor expression
 - d. Aberrant expression of CD5 antigen on CD19⁺ cells

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Myeloproliferative Neoplasms

33

Tim R Randolph

OUTLINE

Chronic Myelogenous Leukemia

Incidence
Cytogenetics of the Philadelphia Chromosome
Molecular Genetics
Pathogenetic Mechanism
Peripheral Blood and Bone Marrow
Other Laboratory Findings
Progression
Related Diseases
Treatment

Polycythemia Vera

Pathogenetic Mechanism
Diagnosis
Peripheral Blood and Bone Marrow
Clinical Presentation
Treatment and Prognosis

Essential Thrombocythemia

Incidence
Pathogenetic Mechanism
Clinical Presentation
Diagnosis
Peripheral Blood and Bone Marrow
Treatment and Prognosis

Primary Myelofibrosis

Myelofibrosis
Hematopoiesis and Extramedullary Hematopoiesis
Pathogenetic Mechanism
Incidence and Clinical Presentation
Peripheral Blood and Bone Marrow
Immune Response
Treatment and Prognosis

Summary of Current Therapy of Non-BCR/ABL1, Primary MPNs

Interconnection among Essential Thrombocythemia, Polycythemia Vera,

OBJECTIVES

After completion of this chapter, the reader will be able to:

1. Define myeloproliferative neoplasms (MPNs), list the most common diseases included in the World Health Organization (WHO) classification of MPNs, and recognize their abbreviations.
2. Define chronic myelogenous leukemia (CML), and describe the cell lines involved, the clinical phases, and the expected clinical manifestations, key peripheral blood and bone marrow findings, and diagnostic criteria applicable to each stage.
3. Discuss the cytogenetics, molecular genetics, and molecular pathophysiology of CML and relate it to treatment approaches, monitoring minimal residual disease, mechanisms of drug resistance.
4. Define polycythemia vera (PV), and describe the cell lines involved, clinical manifestations, key peripheral blood and bone marrow findings, and the diagnostic criteria.
5. Discuss the *JAK2* mutation and the proposed pathogenetic mechanism in PV.
6. Discuss the progression of PV and treatment modalities to include JAK inhibitors.
7. Define essential thrombocythemia (ET), and describe the cell lines involved, clinical manifestations, key peripheral blood and bone marrow findings, and the diagnostic criteria.
8. Discuss common mutations, pathophysiology, and two complications that may occur in patients with ET.
9. Define primary myelofibrosis (PMF), and describe the cell lines involved, clinical manifestations, key pathologic features in peripheral blood, bone marrow, and tissues, and the diagnostic criteria.
10. Describe the mutations that occur in PMF and relate them to disease progression and current therapy.
11. Briefly discuss the potential interrelationships between the mutations and hypotheses for disease development and progression among between ET, PV, and PMF.
12. Briefly describe the other myeloproliferative disorders outlined in this chapter.
13. Given complete blood count and cytogenetic, molecular, and other laboratory results, recognize the findings consistent with each major MPN.
14. Recommend follow-up testing for suspected MPN and interpret the results of testing.

CASE STUDY

After studying the material in this chapter, the reader should be able to respond to the following case study:

A 34-year-old woman came to the physician with a 2-month history of increasing weakness, persistent nonproductive cough, fever and chills accompanied by night sweats, and a 13-pound weight loss over a 6-month period. Results of chest radiographs and purified protein derivative test (for tuberculosis) were negative. The patient was treated with ciprofloxacin and her cough improved, but she continued to grow weaker and was able to consume only small quantities of food. The patient appeared pale and cachectic. Tenderness and fullness were present in the left upper quadrant, and the spleen was palpable below the umbilicus. No hepatomegaly or peripheral adenopathy was noted. Her laboratory results were as follows:

WBCs— $248 \times 10^9/L$

HGB—9.5 g/dL

Continued

OUTLINE—cont'd
and Primary Myelofibrosis
Other Myeloproliferative Neoplasms

Chronic Neutrophilic Leukemia

Chronic Eosinophilic Leukemia, Not Otherwise Specified

Mastocytosis

Myeloproliferative Neo-

CASE STUDY—cont'd

After studying the material in this chapter, the reader should be able to respond to the following case study:

HCT—26.3%

Platelets— $449 \times 10^9/L$

Segmented neutrophils—44%

Band neutrophils—4%

Lymphocytes—10%

Eosinophils—3%

Basophils—7%

Myelocytes—30%

Promyelocytes—1%

Myeloblasts—1%

Nucleated RBCs—2 per 100 WBCs

Reticulocytes—3%

Leukocyte alkaline phosphatase (LAP) score—20 (reference range, 40 to 130)

Lactate dehydrogenase—692 IU (reference range, 140 to 280 IU)

Uric acid—8.1 mg/dL (reference range, 4 to 6 mg/dL)

1. What is the significance of the elevated WBC count and abnormal WBC differential?
2. How does the LAP score aid in the diagnosis?
3. Justify the use of cytogenetic studies in a patient with test results similar to those in this case study.
4. Predict the results of the cytogenetic studies.
5. Describe the molecular mutation resulting from the cytogenetic abnormality.
6. What is the usual treatment for this disorder?
7. Briefly discuss mechanisms of drug resistance.

The myeloproliferative neoplasms (MPNs) are clonal hematopoietic disorders caused by genetic mutations in the hematopoietic stem cells that result in expansion, excessive production, and accumulation of erythrocytes, granulocytes, and platelets. Myeloproliferation is due to hypersensitivity or independence of normal cytokine regulation that reduces cytokine levels through negative feedback systems normally induced by mature cells.^{1,2} Expansion occurs in varying combinations in the bone marrow, peripheral blood, and tissues.³⁻⁶ The MPNs have pathogenetic similarities, as well as common clinical and laboratory features.⁷

MPNs are predominantly chronic with accelerated, subacute, or acute phases. In certain patients it is difficult to make a clear delineation between subacute and chronic phases using clinical and morphologic findings.

The World Health Organization (WHO) has classified the MPNs into four predominant disorders: chronic myelogenous leukemia (CML); polycythemia vera (PV), also known as *polycythemia rubra vera*; essential (primary) thrombocythemia (ET); and primary myelofibrosis (PMF), also known as *agnogenic myelofibrosis with myeloid metaplasia* and *chronic idiopathic myelofibrosis*. Several other less common MPN conditions have been described and are classified as chronic neutrophilic leukemia (CNL); chronic eosinophilic leukemia (CEL), not otherwise specified; mastocytosis; and myeloproliferative disorder, unclassified.⁸ CML and PV are defined by their overproduction of

granulocytes and erythrocytes, respectively.^{4,9,10} PMF is a combination of overproduction of hematopoietic cells and stimulation of fibroblast production leading to ineffective hematopoiesis with resultant peripheral blood cytopenias.¹¹ ET is characterized by increased megakaryocytopoiesis and peripheral blood thrombocytosis.¹²

MPNs present as stable chronic disorders that may transform first to a subacute, then to an aggressive cellular growth phase, such as acute myeloid leukemia (AML) or acute lymphoblastic leukemia (ALL). They may manifest a depleted cellular phase, such as bone marrow hypoplasia, or exhibit clinical symptoms and morphologic patterns characteristic of a subacute followed by a more aggressive cellular expression. Familial MPNs have been described in families in which two or more members are affected.¹³

CHRONIC MYELOGENOUS LEUKEMIA

Chronic myelogenous leukemia (CML) is an MPN arising from a single genetic translocation in a pluripotential hematopoietic stem cell producing a clonal overproduction of the myeloid cell line, resulting in a preponderance of immature cells in the neutrophilic line. CML begins with a chronic clinical phase and, if untreated, progresses to an accelerated phase in 3 to 4 years and often terminates as an acute leukemia. The clinical features are frequent infection, anemia, bleeding, and splenomegaly, all

secondary to massive pathologic accumulation of myeloid progenitor cells in bone marrow, peripheral blood, and extramedullary tissues. Neutrophilia with all maturational stages present, basophilia, eosinophilia, and often thrombocytosis are noted in peripheral blood. The clonal origin of hematopoietic cells in CML has been verified in studies of females heterozygous for glucose-6-phosphate dehydrogenase. Only one isoenzyme is active in affected cells, whereas two isoenzymes are active in nonaffected cells.¹⁴

Incidence

CML occurs at all ages but is seen predominantly in those aged 46 to 53 years. It represents about 20% of all cases of leukemia, is slightly more common in males than in females, and carried a mortality rate of 1.5 per 100,000 per year in the era prior to the development of imatinib mesylate (Gleevec). Imatinib is a tyrosine kinase inhibitor that has changed the prognosis and treatment for CML and is described in detail later.

Symptoms associated with clinical onset are usually of minimal intensity and include fatigue, decreased tolerance of exertion, anorexia, abdominal discomfort, weight loss, and symptomatic effects from splenic enlargement.

Cytogenetics of the Philadelphia Chromosome

A unique chromosome, the Philadelphia chromosome, is present in proliferating hematopoietic stem cells and their progeny in CML and must be identified to confirm the diagnosis. Although the cause of Philadelphia chromosome formation is unknown, it appears more frequently in populations exposed to ionizing radiation.^{15,16} In most patients, a cause cannot be identified. Appearance of the Philadelphia chromosome in donor cells after allogeneic bone marrow transplantation indicates the possibility of a transmissible agent.¹⁷ The Philadelphia chromosome was first identified as a short chromosome 22 in 1960 by Nowell and Hungerford in Philadelphia.¹⁸ In 1973 Rowley, of the University of Illinois at Chicago, discovered that the Philadelphia chromosome is a reciprocal translocation between the long arms of chromosomes 9 and 22 (Chapter 30).¹⁹ This acquired somatic mutation specifically reflects the translocation of an *ABL* proto-oncogene from band q34 of chromosome 9 to the breakpoint cluster region (BCR) of band q11 of chromosome 22, resulting in a unique chimeric gene, *BCR-ABL1*.²⁰ This new gene produces a 210-kD BCR/ABL fusion protein (p210BCR/ABL) that expresses enhanced tyrosine kinase activity from the ABL moiety compared with its natural enzymatic counterpart.

Molecular Genetics

The t(9;22) translocation that produces the *BCR/ABL1* chimeric gene has been observed in four primary molecular forms that produce three versions of the BCR/ABL chimeric protein: p190, p210, and p230 (Figure 33-1). The four genetic variations are based on the area of the *BCR* gene that houses the breakpoint on chromosome 22, because the breakpoint on chromosome 9 occurs in the same location. The wild-type (normal) *ABL1* gene on chromosome 9 is a relatively large gene of approximately

230 kilobases (kb) containing 11 exons. The breakpoint consistently occurs 5' of the second exon such that exons 2 to 11 are contributed to the *BCR/ABL1* fusion gene.²⁰

There are four *BCR* genes in the human genome: *BCR1*, *BCR2*, *BCR3*, and *BCR4*. It is the *BCR1* gene that is involved in the Philadelphia translocation. The wild-type (normal) *BCR1* gene is approximately 100 kb with 20 exons. In 1984 Groffen and colleagues identified the BCR on chromosome 22 as a 5-exon region involving exons 12 to 16 that was the area of breakage in the traditional t(9;22) translocation.²¹ This area was later termed the *major BCR*. Two other areas of breakage were identified on chromosome 22, one near the 5' (head) of the *BCR1* gene, called the *minor BCR*, and one in the 3' end (tail) of the *BCR1* gene, termed the *micro BCR*. Therefore, two areas of breakage in the major BCR, one breakpoint area in the minor BCR, and one breakpoint region in the micro BCR produce four versions of the *BCR* gene that combine with the *ABL1* gene to form four versions of the *BCR/ABL1* chimeric gene.

Within the major BCR two specific breakpoints account for the t(9;22) translocation involved in the development of CML. Breakage in the *BCR1* gene in the major BCR contributes exons 1 to 13 or 1 to 14, whereas the *ABL1* gene contributes exons 2 to 11. Because the two breakpoints in the major BCR differ by only one exon, the chimeric protein product is essentially the same size and is designated as the p210 protein. Breakage in the minor BCR contributes only exon one from *BCR1*, which joins with the same exons 2 to 11 of *ABL1* to produce a p190 protein. The micro BCR breakpoint contributes exons 2 to 19 from *BCR1*, which fuse with *ABL1* exons 2 to 11, producing the p230 protein. Therefore, the four possible *BCR1* breakpoints produce four different chimeric genes, resulting in a total of three different protein products.²²

Pathogenetic Mechanism

To understand the aberrant function of the BCR/ABL fusion protein, it is first helpful to understand both the normal BCR and ABL proteins. The wild-type ABL protein, when in its usual location on chromosome 9, codes for p125, which exhibits normal tyrosine kinase activity. The *BCR1* gene produces p160, expresses serine and threonine kinase activity, and is thought to function in the regulation of cell growth. Protein kinases are enzymes that catalyze the transfer of phosphate groups from adenosine triphosphate (ATP), guanosine triphosphate, and other phosphate donors to receiver proteins. A tyrosine kinase transfers the phosphate group to a tyrosine amino acid on the receiver protein. For the kinase activity of the ABL protein to occur, the ABL protein must first be phosphorylated. This is often accomplished through autophosphorylation. The ABL protein has three primary domains called *SH1*, *SH2*, and *SH3* that together express and regulate the kinase activity. *SH1* is the binding site for ATP; *SH2* is the docking point for phosphate receiver proteins; and *SH3* is the domain that controls the phosphorylation activity. When ATP binds to the ATP binding site, the phosphate is transferred to the *SH2* region of the ABL protein, which initiates a conformational change that alters the tertiary structure of the protein and exposes the active site of the kinase enzyme. When a second ATP binds the ATP binding

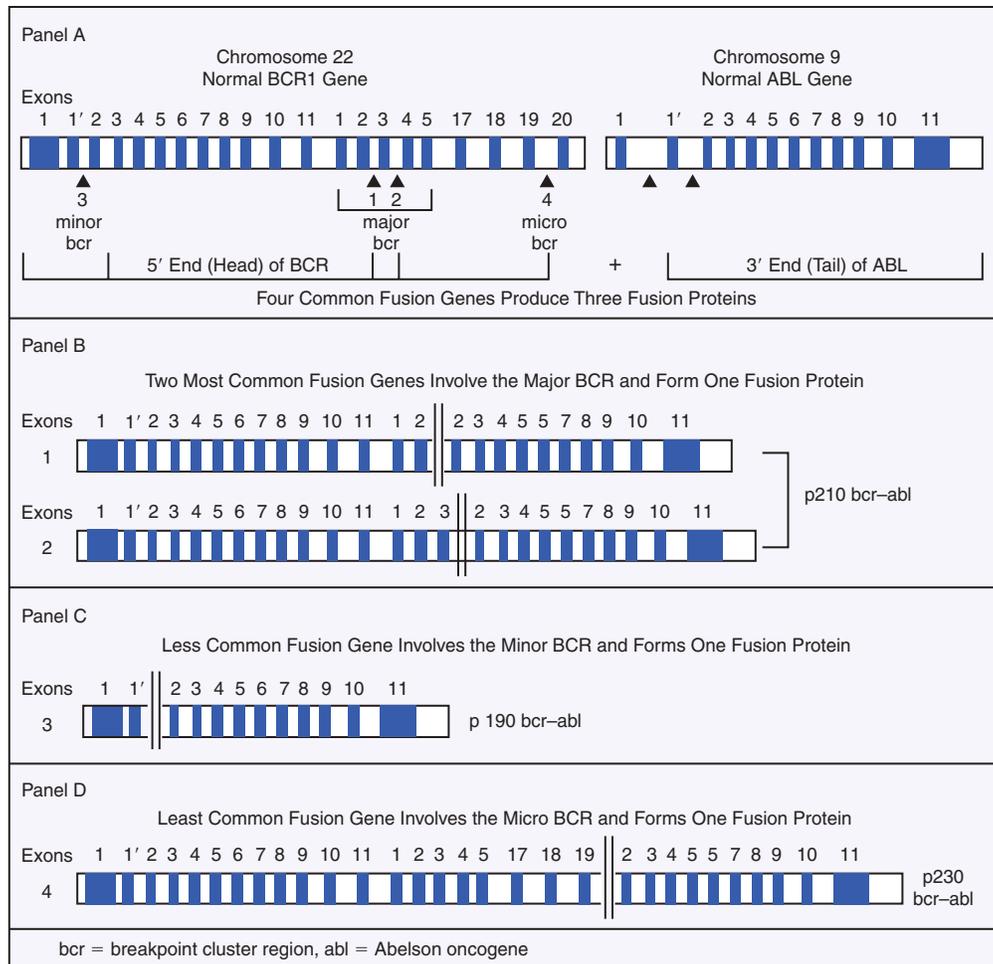


Figure 33-1 Molecular biology of the *BCR/ABL* fusion gene. **A**, Normal *BCR1* gene on chromosome 22 and *ABL* gene on chromosome 9. **B**, Two *BCR* fusion gene products from the major *BCR*. **C**, Fusion gene product from the minor *BCR*. **D**, Fusion gene product from the micro *BCR*.

site and a receiver protein docks in the SH2 domain, the phosphate group is transferred to the receiver protein. In most physiologically normal intracellular pathways, protein phosphorylation activates the receiver proteins (Figure 33-2). This phosphorylation initiates a cascade of phosphorylation events, each activating the next protein until a transcription factor becomes activated. These activation cascades, called *signal transduction pathways*, are designed to activate genes necessary to control cell proliferation, differentiation, and natural cell death, called *apoptosis*. There are several signal transduction pathways activated by the *ABL* tyrosine kinase that function in concert to activate these genes in a precise order and at the required level of activation to control these cellular events.^{22,23}

In the case of CML, the *BCR/ABL1* translocation occurs next to the SH3 domain of the *ABL1* moiety, which is designed to control the rate and timing of phosphorylation. Therefore, the *BCR/ABL* tyrosine kinase loses the ability to shut off kinase activity and is said to have constitutive tyrosine kinase activity. The *BCR/ABL* enzyme continuously adds phosphate groups to tyrosine residues on cytoplasmic proteins, activating several signal transduction pathways. These pathways stimulate gene expression, keeping the myeloid cells proliferating, reducing differentiation, reducing adhesion of cells to bone marrow

stroma, and virtually eliminating apoptosis. The result is increased clonal proliferation of myeloid cells secondary to a reduction in or loss of sensitivity to protein regulators.²⁴ There is an increase in growth factor-independent cellular proliferation from activation of the *RAS* gene and a decrease in or resistance to apoptosis. New clones of stem cells vulnerable to additional genetic changes lead to the accelerated and blast phases of CML. In addition, the *BCR/ABL* protein localizes in the cytoplasm rather than in the nucleus, as does the normal *ABL* protein. The mutation affects maturation and differentiation of hematopoietic and lymphopoietic cells, whose progeny eventually dominates in the affected individual. Progeny cells that exhibit this chromosome include neutrophils, eosinophils, basophils, monocytes, nucleated erythrocytes, megakaryocytes, and B lymphocytes.^{9,25}

In addition, the loss of genetic segments in the 5' end of the *ABL1* gene results in an altered protein-binding affinity for F-actin, which leads to a reduction in contact binding of hematopoietic CML cells to stromal cells, causing premature release of cells into the circulation.²³ Abnormal adhesion between stem cells and stroma may dysregulate hematopoiesis. One action of interferon- α therapy is to reverse the loss of adhesion of CML progenitor cells, which reduces the premature release of these cells into the circulation.²⁶

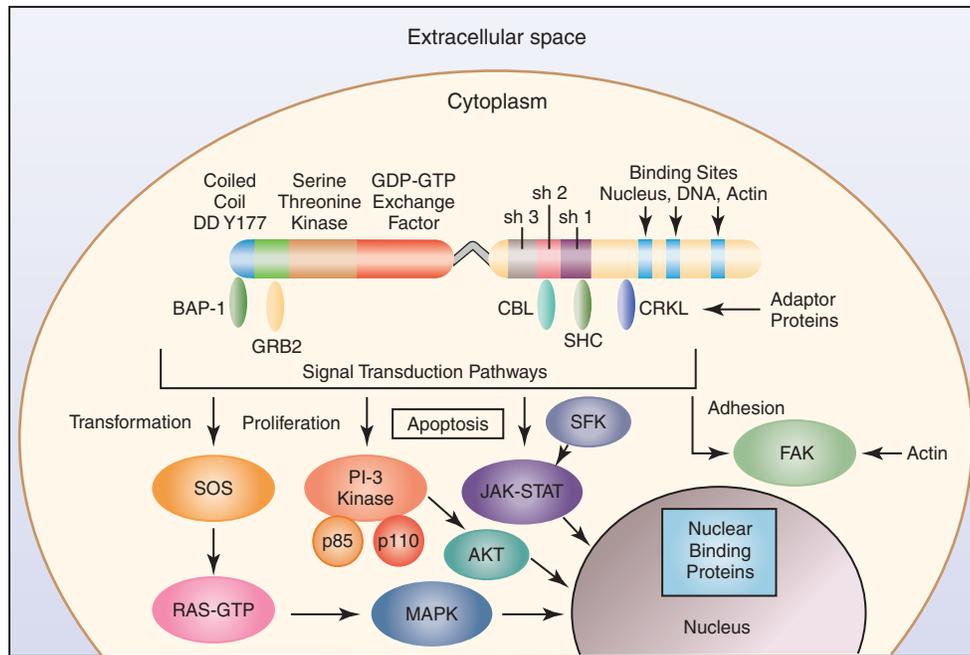


Figure 33-2 Signal transduction pathways influenced by the BCR/ABL fusion protein.

Apoptotic functions are lost because the BCR/ABL fusion protein has a propensity to be sequestered in the cytoplasm, which has antiapoptotic functions. The p210 is necessary for CML transformation of the hematopoietic stem cell.

The *BCR/ABL1* fusion gene is also identified with Philadelphia chromosome–positive ALL. The chromosome appears in 20% of adults and 2% to 5% of children with this disease. The minor chimeric *BCL/ABL1* gene that transcribes and translates to a p185/p190 protein is present in 50% of Philadelphia chromosome–positive ALL cases in adults and 75% of Philadelphia chromosome–positive ALL cases in children. The micro *BCR*, when fused with the *ABL1* gene, produces a large p230 protein that is associated with chronic neutrophilic leukemia and is the least common version found.

Peripheral Blood and Bone Marrow

There are dramatic morphologic changes in the peripheral blood and bone marrow that reflect the expansion of the granulocyte pool, particularly in the later maturational stages. [Table 33-1](#) lists the qualitative changes in the peripheral blood, bone marrow, and extramedullary tissues that are commonly observed at the time of diagnosis. A dramatic left shift is noted that extends down to the promyelocyte stage and occasionally even produces a few blasts in the peripheral blood. The platelet count is often elevated, reflecting the myeloproliferative nature of the disease. Extramedullary granulopoiesis may involve sinusoids and medullary cords in the spleen and sinusoids, portal tract zones, and solid areas of the liver.

[Figure 33-3](#) illustrates a common pattern in the peripheral blood film of chronic phase CML at the time of diagnosis. Leukocytosis is readily apparent at scanning microscopic powers. Segmented neutrophils, bands, metamyelocytes, and myelocytes predominate, and immature and mature eosinophils

and basophils are increased. Myeloblasts and promyelocytes are present at a rate of approximately 1% and 5%, respectively. Lymphocytes and monocytes are present and often show an absolute increase in number but a relative decrease in percentage. Nucleated red blood cells (NRBCs) are rare. Platelets are normal or increased, and some may exhibit abnormal morphology.

Bone marrow changes are illustrated in [Figure 33-4](#). An intense hypercellularity is present due to granulopoiesis, marked by broad zones of immature granulocytes, usually perivascular or periosteal, differentiating into more centrally placed mature granulocytes. Normoblasts appear reduced in number. Megakaryocytes are normal or increased in number and, when increased, may appear in clusters and exhibit dyspoietic cytologic changes. They often appear small with reduced nuclear size (by approximately 20%) and reduced nuclear lobulations. Reticulin fibers are increased in approximately 20% of patients. Increased megakaryocyte density is associated with an increase in myelofibrosis.²⁷ The presence of pseudo-Gaucher cells (Chapter 29) usually occurs.

Other Laboratory Findings

Hyperuricemia and uricosuria from increased cell turnover may be associated with secondary gout, urinary uric acid stones, and uric acid nephropathy.²⁸ Approximately 15% of patients exhibit total white blood cell (WBC) counts greater than $300 \times 10^9/L$.²⁹ Symptoms in these patients are secondary to vascular stasis and possible intravascular consumption of oxygen by the leukocytes. Symptoms are reversible with the lowering of the total WBC count.³⁰

In patients with the typical peripheral blood findings discussed above, the diagnosis of CML is confirmed by demonstrating the presence of the t(9;22) translocation by cytogenetic

TABLE 33-1 Common Morphologic Changes in Chronic Myelogenous Leukemia

Peripheral Blood	
Erythrocytes	Normal or decreased
Reticulocytes	Normal
Nucleated red blood cells	Present
Total white blood cells	Increased
Lymphocytes	Normal or increased
Neutrophils	Increased
Basophils	Increased
Eosinophils	Increased
Myelocytes	Increased
Leukocyte alkaline phosphatase	Decreased
Platelets	Normal or increased
Bone Marrow	
Cellularity	Increased
Granulopoiesis	Increased
Erythropoiesis	Decreased
Megakaryopoiesis	Increased or normal
Reticulin	Increased
Macrophages	
Gaucherlike	Sea blue
Green-gray crystals	Increased
Megakaryocytes	
Small	Increased
Extramedullary Tissue	
Splenomegaly	Present
Sinusoidal	Present
Medullary	Present
Hepatomegaly	Present
Sinusoidal	Present
Portal tract	Present
Local infiltrates	Present

analysis (Figure 30-1), detection of the *BCR/ABL1* fusion gene using fluorescence in situ hybridization (Figures 30-2 and 30-21), and/or detecting the *BCR/ABL1* fusion transcript by qualitative reverse transcriptase polymerase chain reaction (Figure 31-12).

Although molecular techniques are more commonly used to diagnose CML, initial testing of the cells for leukocyte alkaline phosphatase (LAP) enzyme activity may be useful in some setting for preliminary differentiation of CML from a leukemoid reaction due to severe infections (Chapter 29).

LAP is an enzyme found in the membranes of secondary granules of neutrophils. In the procedure a blood film is incubated with a naphthol-phosphate substrate and diazo dye at an alkaline pH. The LAP enzyme hydrolyzes the substrate, and the liberated naphthol reacts with the dye producing a colored precipitate on the granules. The slide is examined microscopically and 100 segmented neutrophils and bands are counted and rated from 0 to 4+ based on the intensity of

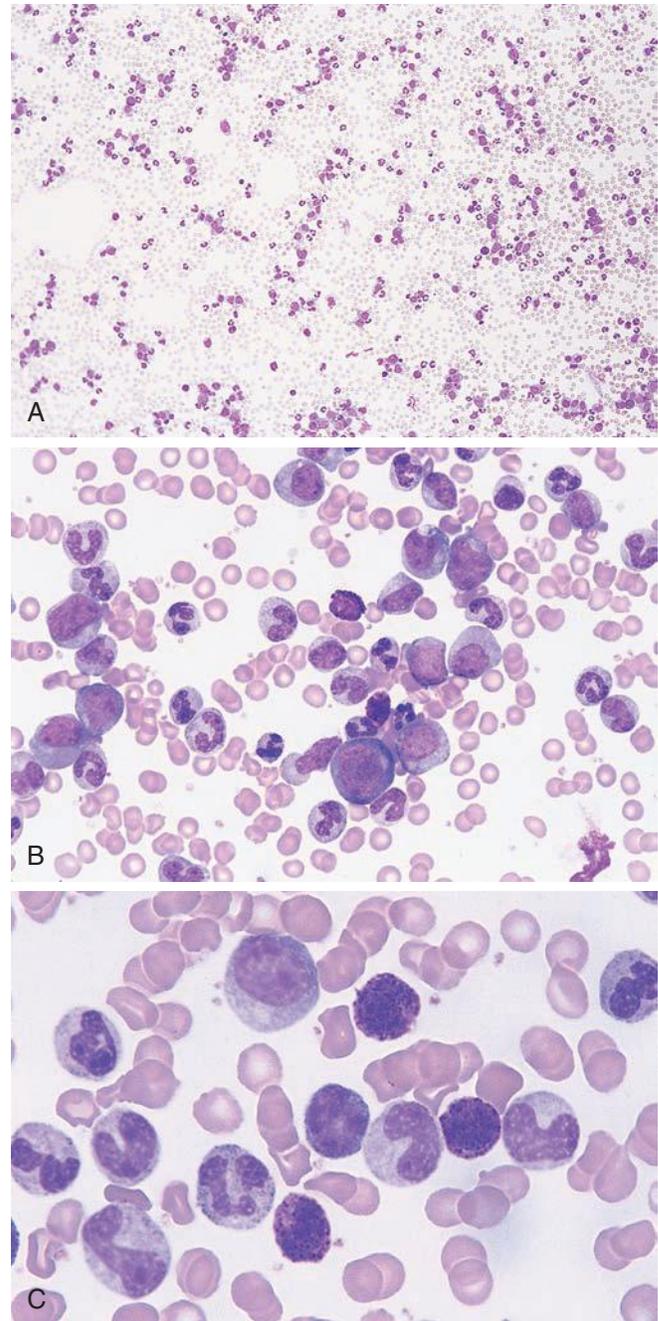


Figure 33-3 Peripheral blood films in the chronic phase of chronic myelogenous leukemia. **A**, Leukocytosis is evident at scanning power ($\times 100$). **B**, Bimodal population of segmented neutrophils and myelocytes ($\times 500$). **C**, Increased basophils and immature neutrophils ($\times 1000$).

the staining. The LAP score is calculated by multiplying each score by the number of cells, and adding the products. For example, 5 cells with 4+ staining, 5 cells with 3+, 25 cells with 2+, 45 cells with 1+, and 20 cells with 0 staining calculates to a LAP score of 130. Because scoring is subjective, the mean score of two examiners is reported, and they should agree within 10%.

A sample reference interval for the LAP score is 15 to 170, but every laboratory establishes its own. The LAP score is decreased in untreated CML, and normal or increased in

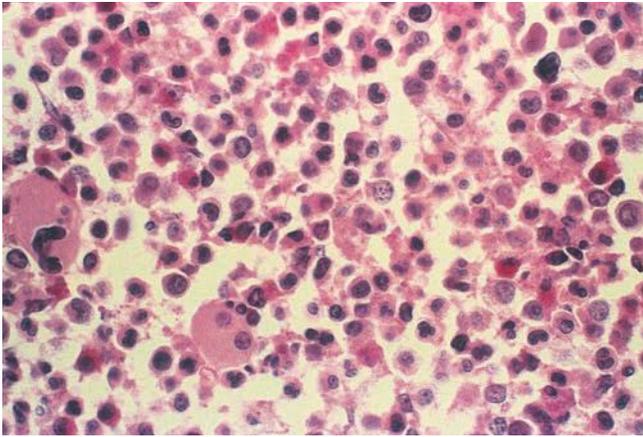


Figure 33-4 Bone marrow biopsy specimen in the chronic phase of chronic myelogenous leukemia, showing hypercellularity with increased granulocytes and megakaryocytes (hematoxylin and eosin stain, $\times 400$).

leukemoid reactions. Individuals with polycythemia vera or those in the third trimester of pregnancy also have higher LAP scores.

Progression

In the pre-imatinib era, most cases of this disease would eventually transform into acute leukemia.³¹ Before blastic transformation, some patients proceed through an intermediate *metamorphosis* or *accelerated* phase. Disease progression is accompanied by an increase in the frequency and number of clinical symptoms, adverse changes in laboratory values, and poorer response to therapy than in the chronic phase. Additional chromosome abnormalities reflect evolution of the malignant clone and may appear, associated with enhanced dyshematopoietic cell maturation patterns and increases in morphologic and functional abnormalities in blood cells. There is often an increasing degree of anemia and, in the peripheral blood, fewer mature leukocytes, more basophils, and fewer platelets, with a greater proportion of abnormal platelets, micromegakaryocytes, and megakaryocytic fragments. The circulating blast count increases to 10% to 19%. This total blast percentage, or a combination of 20% blasts and promyelocytes, has been proposed as a diagnostic criterion for the accelerated phase.³²

Blast crisis involves the peripheral blood, bone marrow, and extramedullary tissues. Based on acute leukemia definitions, blasts constitute more than 20% of total bone marrow cellularity, and the peripheral blood exhibits increased blasts.³¹ Blast crisis leukemia usually is AML or ALL, but origins from other hematopoietic clonal cells are possible. Extramedullary growth may occur as lymphocytic or myelogenous cell proliferations; the latter are often referred to as *granulocytic sarcoma*. Extramedullary sarcoma is observed at many sites or locations in the body and may precede a marrow blast crisis. The clinical symptoms of blast crisis mimic those of acute leukemia, including severe anemia, leukopenia of all WBCs except blasts, and thrombocytopenia. Chromosome abnormalities such as additional Philadelphia chromosome(s), isochromosome 17, trisomy 8, loss of Y chromosome, and trisomy 19 accumulate with disease progression.^{33,34} These

generally occurred in approximately 75% of patients in the pre-imatinib era.

Related Diseases

Several diseases exist that are clinically similar to CML but do not exhibit the Philadelphia chromosome and express only a few pseudo-Gaucher cells. Chronic neutrophilic leukemia is another MPN that manifests with peripheral blood, bone marrow, and extramedullary infiltrative patterns similar to those of CML, except that only neutrophilic granulocytes are present and fewer than 10% of peripheral blood neutrophils are immature.³⁵ Similarly, chronic monocytic leukemia involves a comparable expansion of monocytes, including functional monocytes.³⁶

Juvenile myelomonocytic leukemia and adult chronic myelomonocytic leukemia are classified by the WHO as myelodysplastic/myeloproliferative diseases because of the overlap in clinical, laboratory, or morphologic findings. Juvenile myelomonocytic leukemia is observed in children younger than 4 years of age and is accompanied by an expansion in the number of monocytes and granulocytes, including immature granulocytes, and manifestations of dyserythropoiesis.³⁷

The peripheral blood of adults with chronic myelomonocytic leukemia may have characteristics similar to those seen in the refractory anemias, such as oval macrocytes and reticulocytopenia. The peripheral WBC concentration may reach $100 \times 10^9/L$. According to WHO criteria, absolute monocytosis (more than 1×10^9 monocytes/L) must be present to make the diagnosis. Clinical features include prominent splenomegaly, symptoms of anemia, fever, bleeding, and infection. Before the presence of the Philadelphia chromosome was established as a requirement for the diagnosis of CML, some cases that were classified as Philadelphia chromosome–negative CML likely represented misdiagnoses of chronic myelomonocytic leukemia.³⁸ Chronic myelomonocytic leukemia is discussed further with myelodysplastic syndromes in Chapter 34.

A puzzling group of patients exhibit Philadelphia chromosome–positive acute leukemia. Studies reveal that 2% of patients with AML exhibit Philadelphia chromosome in a significant proportion of blasts. Further, 5% of patients with childhood-onset ALL and 20% of those with adult-onset ALL test positive for Philadelphia chromosome.³⁹⁻⁴² The proper alignment of these cases within the spectrum of CML is speculative. It is understood that some of these cases likely represent undiagnosed CML that rapidly progressed to an acute leukemia prior to diagnosis. However, because rapidly dividing malignant cells are more prone to genetic mutation, the presence of the Philadelphia chromosome in acute leukemias may reflect a late-stage mutation that contributed little to acute leukemia leukemogenesis.

Treatment

Early treatment approaches for CML were unable to produce remission, so the goal of therapy became the reduction of tumor burden. The first forms of therapy for CML included alkylating agents such as nitrogen mustard,⁴³ introduced in the late 1940s, and busulfan,⁴⁴ which came into use in the early 1950s. Later, busulfan in combination with 6-thioguanine was used to achieve the goal of tumor burden reduction. Other drugs like

hydroxyurea and 6-mercaptopurine were introduced later and found to improve patient survival. The discovery of interferon- α in 1983 dramatically improved outcomes of patients with CML by inducing the suppression of the Philadelphia chromosome, reducing the rate of cellular progression to blast cells, and increasing the frequency of long-term patient survival.⁴⁵

Interferon- α stimulates a cell-mediated antitumor host response that reduces myeloid cell numbers, induces cytogenetic remissions, and increases survival.⁴⁶ It improves the frequency and duration of hematologic remission and reduces the frequency of detection of the Philadelphia chromosome. In some patients, a complete cytogenetic remission is achieved for a time.

In 1997 it was discovered that cytarabine given with interferon- α improved the frequency of hematologic remissions but did not eliminate the *BCR/ABL1* gene, which was still detected by molecular and fluorescent methods.⁴⁷ Also, in some patients the side effects of therapy became severe, drug resistance appeared, and relapse rates were not improved compared with other chemotherapies.

Bone marrow and stem cell transplantation with either autologous or allogeneic hematopoietic stem cells have been reported as curative, especially in patients younger than age 55. Relapses occur, but long-term, disease-free survival is possible. Optimal survival occurs when the patient is treated during the chronic phase within 1 year of diagnosis and is younger than age 50. Treatment requires ablative chemotherapy followed by transplantation of mobilized normal progenitor cells that exhibit CD34+ surface markers. Allogeneic bone marrow transplants are more successful in patients up to age 55 when donors are matched for HLA antigens A, B, and

DR. Donor-matched lymphocyte infusions after allogeneic transplantation of marrow from a sibling donor may assist in producing complete remissions.⁴⁸

Modern therapies involve the use of synthetic proteins that bind the abnormal BCR/ABL protein, blocking the constitutive tyrosine kinase activity and reducing signal transduction activation. Imatinib mesylate is a synthetic tyrosine kinase inhibitor designed to selectively bind the ATP binding site and thus inhibit the tyrosine kinase activity of the BCR/ABL fusion protein. When imatinib binds the ATP binding site, ATP is unable to bind to provide the phosphate group necessary for kinase activity. Imatinib binds the BCR/ABL protein in the inactive conformation, which precedes the autophosphorylation necessary to generate the kinase active site (Figure 33-5).⁴⁹

Goals of therapy include complete hematologic, cytogenetic, and molecular remission indicated by a normalized CBC and differential, absence of Ph1 by karyotype analysis, and absence of measurable BCR/ABL transcripts, respectively. Complete remission from imatinib therapy is induced in part by the reactivation of apoptotic pathways.⁵⁰ The effectiveness of imatinib therapy and stem cell transplantation is best monitored by measuring BCR/ABL transcripts using quantitative real-time reverse transcriptase polymerase chain reaction (RT-PCR). These monitoring tools are used to determine the extent of molecular remission. The most sensitive measure of the effectiveness of imatinib therapy is the number of log reductions of *BCR/ABL* transcripts using real-time RT-PCR.⁵¹ Remission milestones indicating effective imatinib therapy are complete hematologic remission in 3 to 6 months, complete cytogenetic response in 6 months to 1 year, and a 2- to 3-log reduction in

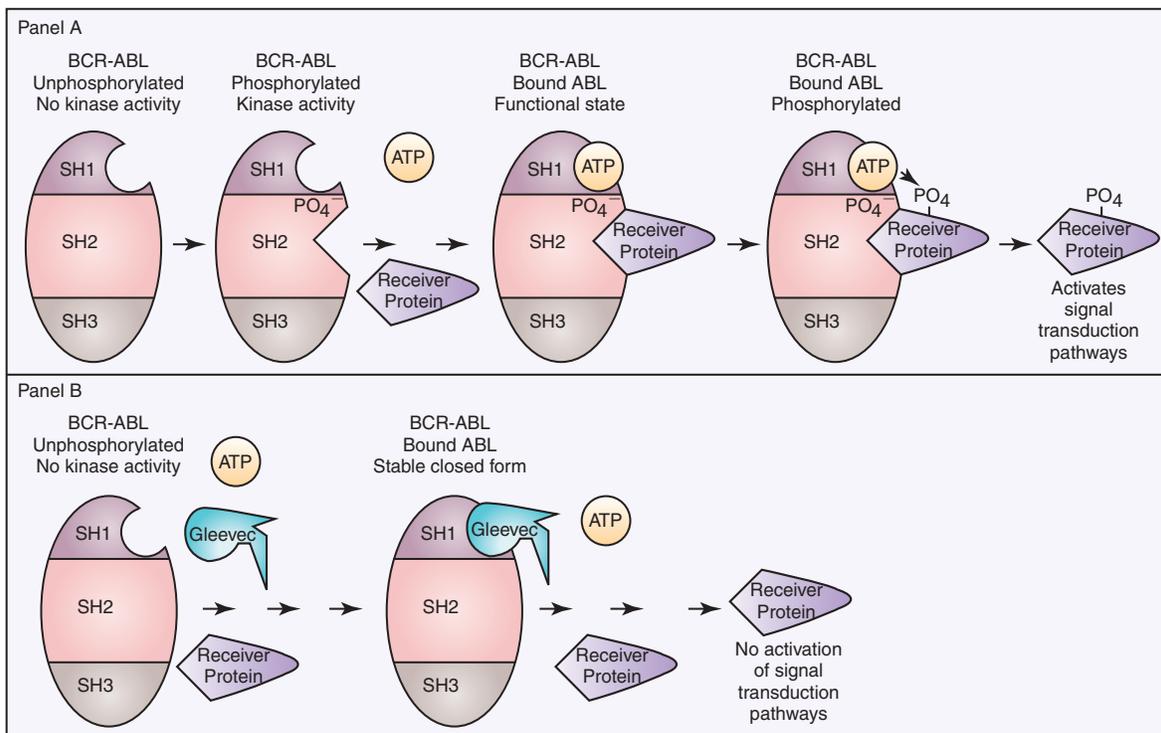


Figure 33-5 Mechanism of imatinib mesylate inhibition of BCR/ABL tyrosine kinase activity. **A**, Mechanism of tyrosine kinase activity of the BCR/ABL fusion protein. **B**, Mechanism of tyrosine kinase inhibition by imatinib mesylate.

BCR/ABL transcripts. When real-time RT-PCR is used, the greatest log reduction possible is a more than 4 log reduction, which represents the maximum sensitivity of the assay. However, discontinuation of imatinib therapy in patients who achieve a more than 4-log reduction usually results in relapse.

Although imatinib has proven to be a successful form of therapy, a major limitation is the development of imatinib resistance resulting in relapse. Approximately 25% to 30% of patients with newly diagnosed CML will discontinue imatinib therapy within 5 years due to lack of remission, resistance, or toxicity.⁵² The two major categories of imatinib resistance are primary and secondary. Primary resistance is defined as the inability to reach the remission milestones. This form of resistance accounts for most treatment failures and probably results from the presence of mutations other than the *BCR/ABL1* mutation at the time of diagnosis. Secondary resistance involves the loss of a previous response and occurs at a rate of 16% at 42 months. The majority of cases of imatinib resistance result from two primary causes: acquisition of additional *BCR/ABL1* mutations and expression of point mutations in the ATP binding site. Additional *BCR/ABL1* mutations can occur through the usual translocation of the remaining unaffected chromosomes 9 and 22, which converts the hematopoietic stem cell from heterozygous to homozygous for the *BCR/ABL1*

can also be acquired from gene duplication during mitosis and accounts for 10% of secondary mutations. An additional *BCR/ABL1* mutation will double the tyrosine kinase activity, making the imatinib dosage inadequate. In these cases higher doses of imatinib will restore remission in most patients (Figure 33-6). The majority of patients who do not respond to higher doses of imatinib express point mutations in the ATP binding site. Over 60 mutations have been identified in the ATP binding site, and these account for the remaining 50% to 90% of secondary mutations. Mutations in the ATP binding site reduce the binding affinity of imatinib, producing some level of resistance (Figure 33-7). Three second-generation tyrosine kinase inhibitors—dasatinib (Sprycel), nilotinib (Dasigna), and bosutinib (Bosulib)—overcome the ATP binding site mutations because they have a much higher binding affinity than imatinib. All three are FDA approved for first-line therapy and are effective at rescuing patients resistant to imatinib, except patients who have developed the T315I mutation. The T315I mutation places a large, bulky isoleucine residue in the center of the ATP binding site, and all four FDA-approved tyrosine kinase inhibitors are resistant to this mutation. However, a third-generation tyrosine kinase inhibitor, ponatinib (Iclusig), inhibits the T315I mutation as well as drugs designed to bind the A loop (receiver protein binding site) will also inhibit tyrosine kinase activity and

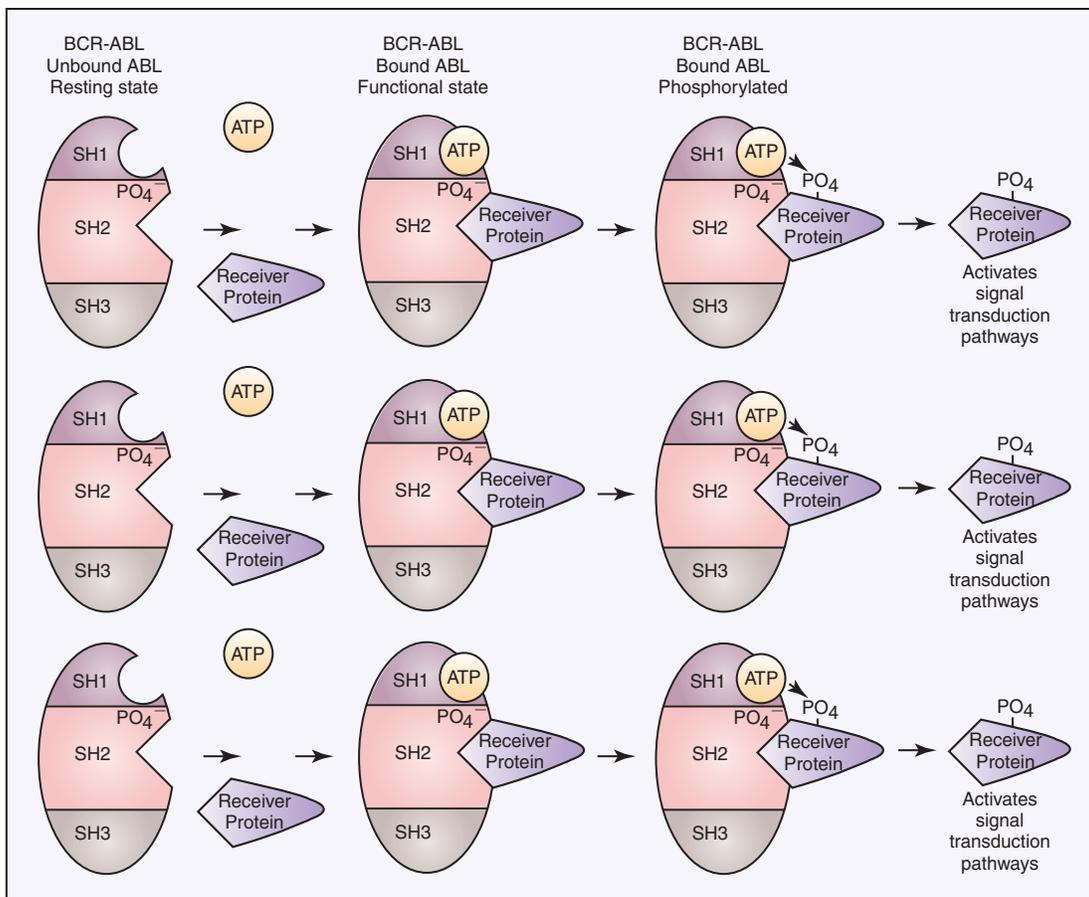


Figure 33-6 Mechanism of imatinib mesylate resistance due to an increased copy number of *BCR/ABL* genes. The increased copies produce more *BCR/ABL* fusion proteins, which results in an increased tyrosine kinase activity requiring a higher dosage of imatinib to restore remission.

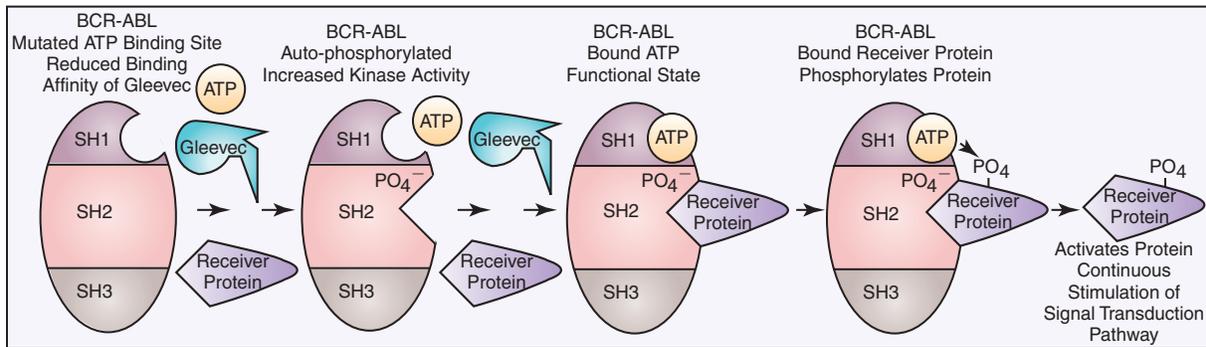


Figure 33-7 Mechanism of imatinib mesylate resistance due to point mutations in the adenosine triphosphate (ATP) binding site. The mutations reduce the binding affinity of imatinib, allowing ATP to bind; this restores the increased tyrosine kinase activity that drives the phenotype and pathogenesis of the disease.

overcome the T315I mutation.⁵³⁻⁵⁵ Currently, studies are under way to evaluate modification of the dosage of imatinib used, to identify and develop other tyrosine kinase inhibitors, and to discover new classes of inhibitors that may be more effective than currently known tyrosine kinase inhibitors.

The development of a care plan for treating a patient with newly diagnosed CML is an ongoing commitment requiring not only the formulation of alternative approaches to achieve and maintain complete cellular remission but also the establishment of laboratory monitoring parameters to follow that confirm long-term success of therapy. Historically, chemotherapy has provided cellular remission but usually has not prevented clinical progression to accelerated or blast phases. Bone marrow transplantation for patients who qualify is likely the preferred choice, but the long-term success (cure) rate remains at 50% to 70%, and most patients will not qualify. For a patient to qualify for transplantation, the patient must be younger than 50 years of age, in the first year of the disease, and have CML that is still in the chronic phase, and a histocompatible donor must be available. For the past 15 years, imatinib has been considered first-line therapy for all patients with newly diagnosed CML. For the small subset of patients who qualify for hematopoietic stem cell transplantation, imatinib is used to induce hematologic remission prior to transplantation. For all other CML patients, imatinib has been used as first-line therapy unless remission is not achieved (primary resistance) or until relapse occurs following remission (secondary resistance). Once the cause of relapse has been determined by cytogenetic and molecular testing, either a higher dosage of imatinib can be given (for an additional *BCR/ABL1* mutation) or a second- or third-generation tyrosine kinase inhibitor (dasatinib, nilotinib, bosatinib) can be prescribed, unless the mutation is the T315I mutation in the ATP binding site. If the T315I mutation is detected, the patient can be given Ponatinib or an A-loop inhibitor (ONO12380) or other drugs like Omacetaxine, MK 0457, or BIRB-796 that inhibit the T315I mutation.⁵⁴ Physicians are beginning to prescribe dasatinib, nilotinib and bosutinib as first-line therapy to replace imatinib in hopes that tyrosine kinase inhibitors with higher binding affinities will extend remissions by reducing the rate of mutation-induced relapses. Among the second-generation TKIs, bosutinib shows the most promise because it demonstrates high potency, has the ability to overcome

most P-loop mutations (except T315I), and shows fewer side effects like neutropenia, thrombocytopenia, cardiotoxicity, and pancreatitis compared to nilotinib and dasatinib. Ponatinib and A-loop inhibitors can be used to rescue patients treated with second-generation tyrosine kinase inhibitors, particularly those who develop the T315I mutation.⁵²

POLYCYTHEMIA VERA

Polycythemia vera (PV) is a neoplastic clonal myeloproliferative disorder that commonly manifests with panmyelosis in the bone marrow and increases in erythrocytes, granulocytes, and platelets in the peripheral blood.² Splenomegaly is common. The disease arises in a hematopoietic stem cell. The hypothesis of a clonal origin for PV is supported by studies of X-linked restriction fragment-length deoxyribonucleic acid (DNA) polymorphisms that demonstrate monoclonal X chromosome inactivation in all blood cells.⁵⁶

Pathogenic Mechanism

In PV, neoplastic clonal stem cells are hypersensitive to, or function independently of, erythropoietin for cell growth. Trace levels of erythropoietin in serum stimulate the growth of erythroid progenitor cells in *in vitro* colony-forming growth systems. There is preservation of hypersensitive and normosensitive erythroid colony-forming units, however, which indicates some level of normal hematopoiesis.⁵⁷ Adverse clinical progression seems to correlate with the propagation of the erythropoietin-sensitive colony-forming units.⁵⁸

Understanding of the pathologic mechanism explaining this phenomenon in PV was significantly advanced in 2005 with the discovery of a consistent mutation in the *JAK2* gene. The specific *JAK2* mutation, *JAK2* V617F, is detected in 90% to 97% of patients with PV. Shortly after the *JAK2* V617F mutation was reported, several groups corroborated the finding using other approaches and showed that the mutation is acquired, clonal, present in the hematopoietic stem cell, constitutively active, and capable of activating the erythropoietic signal transduction pathway in the absence of erythropoietin.^{59,60} The point mutation replaces guanine with thymine at exon 14 of the gene, which changes the amino acid at position 617 from valine to phenylalanine. This one amino acid change prevents the inhibition conformation of the tyrosine

kinase, causing it to remain in the active conformation. More specifically, the phenylalanine mutation in the kinase domain is unable to bind the corresponding amino acid in the pseudokinase domain, as can the valine in the wild-type counterpart, which prevents the protein from folding into the inactive conformation (Figure 33-8).^{61,62}

Normally, erythropoietin is released from the kidney into the blood in response to hypoxia and binds to erythropoietin receptors on the surface of erythroid precursor cells. The resulting conformational change in the erythropoietin receptor causes two erythropoietin receptors to dimerize. This produces a docking point for the head of the inactive *JAK2* protein at a domain known as FERM (Band-4.1, ezrin, radixin, and moesin). Docking of *JAK2* stimulates a phosphorylation event, causing a conformational change, and the valine releases from the pseudokinase domain, converting it to an active tyrosine kinase. *JAK2* can also bind to several other receptors to include MPL (myeloproliferative leukemia, aka TPO-R [thrombopoietin receptor]), GCSF-R (granulocyte colony stimulating factor

receptor), prolactin receptor, growth hormone receptor, GM-CSF-R (granulocyte/monocyte colony stimulating factor receptor), IL-3-R (interleukin-3 receptor), IL-5-R (interleukin-5 receptor), and INF- γ 2-R (interferon gamma 2 receptor).⁶² The diversity in ligand receptor binding explains the range of myeloid proliferation observed in PV (erythroid), ET (thrombopoietic), and neutrophilic (PMF). Once activated, *JAK2* phosphorylates several cytoplasmic proteins, but the STAT (signal transducer and activator of transcription) proteins are the main targets. A cascade of phosphorylation reactions through the STAT proteins produce activated transcription factors that activate a host of genes designed to drive and control cell proliferation and differentiation while also initiating apoptosis (Figure 33-9). Constitutive tyrosine kinase activity of the *JAK2* protein causes continuous activation of several signal transduction pathways that are normally activated following erythropoietin stimulation via the erythropoietic receptor. Active *JAK2* will phosphorylate STAT proteins in the absence of erythropoietin or will overphosphorylate in its presence (Figure 33-10).

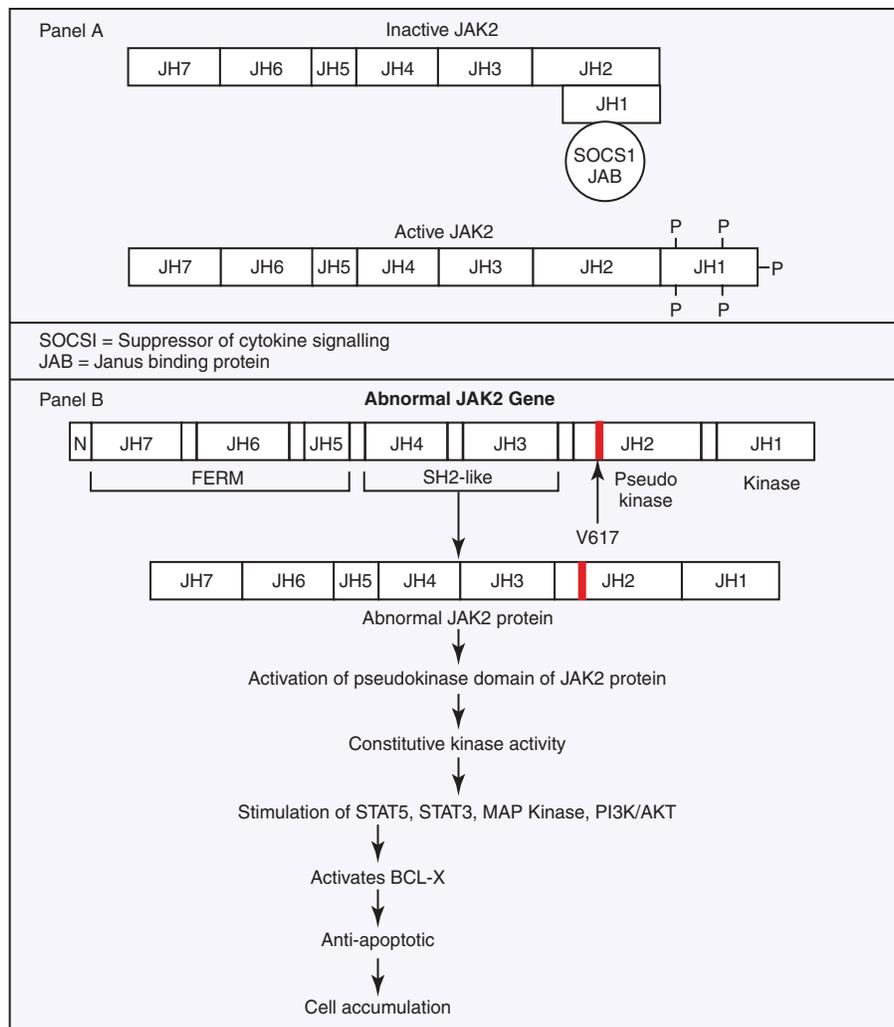


Figure 33-8 Normal regulation of *JAK2* function and loss of regulation from the *JAK2* V617F mutation. **A**, Normal function of the *JAK2* protein and regulation of phosphorylation by *JAK2* intrachain folding and the binding of *JAK2* inhibitors SOCS1 and JAB proteins. **B**, The *JAK2* V617F mutation and the loss of normal *JAK2* folding and inhibitor binding resulting in phosphorylation, activation, and the stimulation of STAT, MAP kinase, and PI3K/AKT signal transduction pathways.

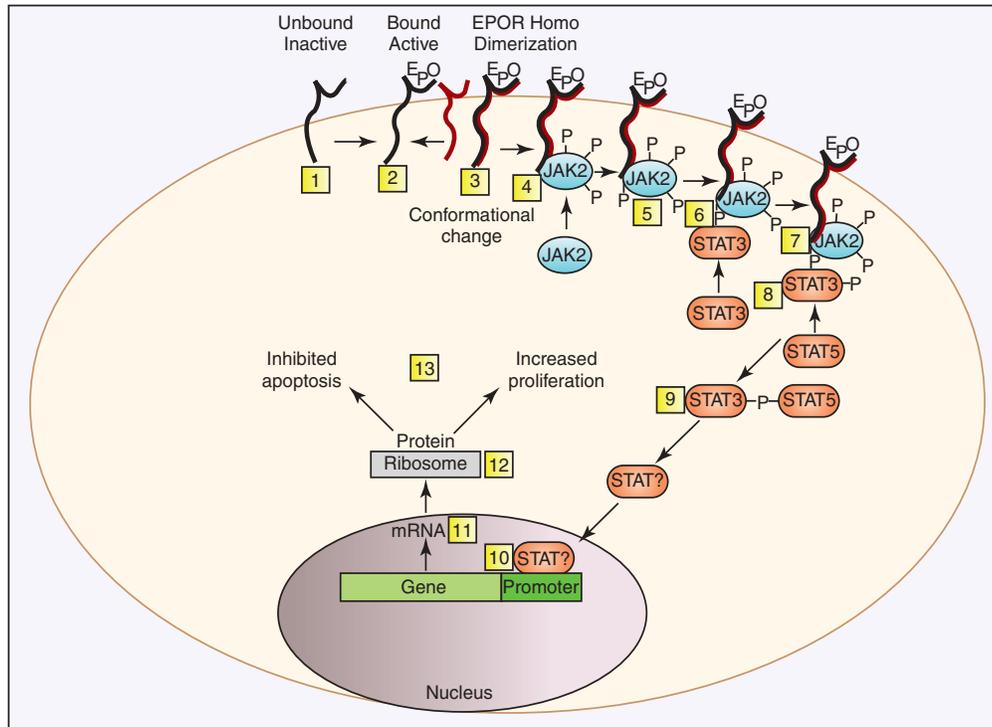


Figure 33-9 Normal erythropoiesis involving erythropoietin binding to erythropoietin receptors and stimulation of the JAK/STAT pathway via the normal *JAK2* protein.

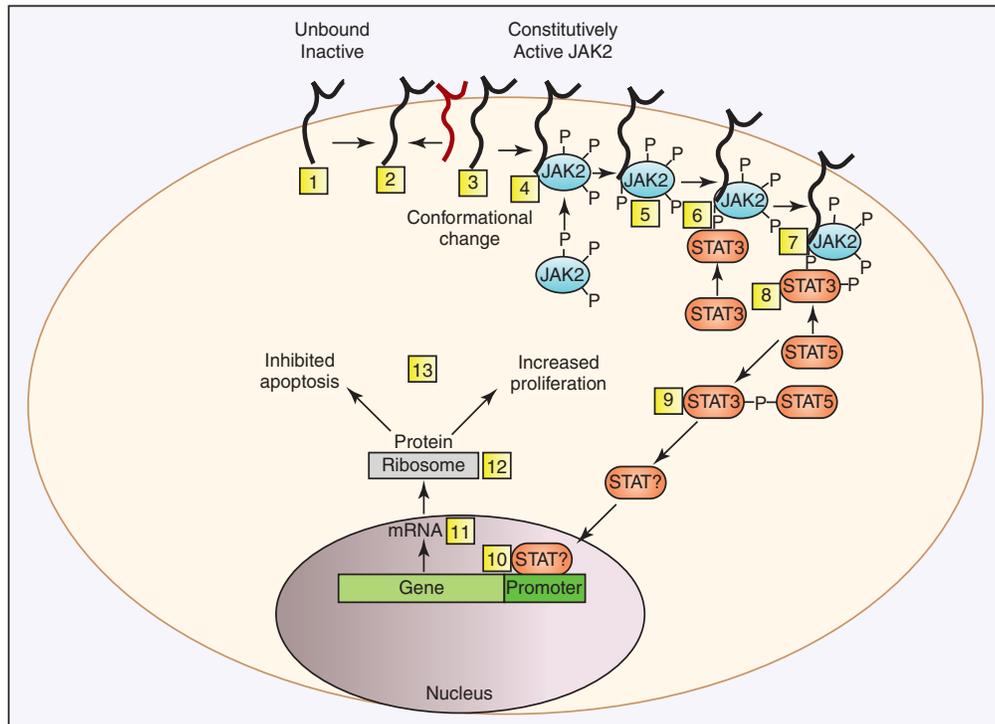


Figure 33-10 Stimulation of erythropoiesis in the absence of erythropoietin that is driven by a constitutively phosphorylated and activated *JAK2* protein resulting from the *JAK2* V617F mutation.

Hematopoietic stem cells that bear the *JAK2* mutation are resistant to erythropoietin-deprivation apoptosis by upregulation of BCL-X, an antiapoptotic protein. PV progenitor cells do not divide more rapidly but accumulate because they do not die normally.⁶³⁻⁶⁵ In addition to the role of mutated *JAK2* in the abrogation of STAT signaling, it has also been shown to

influence chromatin structure^{66,67} and to decrease methyltransferase activity.⁶⁸ Lastly, homozygosity of *JAK2* V617F occurs more commonly in PV, whereas *JAK2* heterozygosity occurs more commonly in ET. Therefore, disease progression from ET to PV may partially be explained by the dosage effect of *JAK2* mutations.⁶²

Because approximately 5% of PV patients do not possess the *JAK2* V617F mutation and because PV has a familial predisposition, it is thought that other mutations must be involved in the pathogenesis of PV and some must precede and possibly predispose the *JAK2* V617F mutation. Since the original discovery of a mutation in the thrombopoietin receptor gene *MPL* in 2006,⁶⁹ several gain-of-function mutations have been identified. Most *MPL* mutations occur in exon 10 where tryptophan 515 is substituted for a leucine, lysine, asparagine, or alanine.⁶⁹⁻⁷² Tryptophan 515 is located on the cytosolic side of the membrane and is key in transducing the signal that thrombopoietin (TPO) has bound to the receptor. These mutations cause the *MPL* receptor to be hypersensitive to TPO and in some cases to assume the active conformation in the absence of TPO. A similar mutation, *MPL* S505N, was initially described in familial PV but has since been found in sporadic MPN.^{69,72} These types of *MPL* mutations have since been identified in up to 15% of *JAK2* V617F-negative ET and PMF patients.⁷³

In 2007 a second type of *JAK2* mutation was identified in exon 12, usually between amino acid residues 536 and 547, that also resulted in a gain of function similar to the *JAK2* V617F.⁷⁴ Exon 12 is not located in the pseudokinase domain, but it is hypothesized that the mutation can modify the structure of the JH2 domain, rendering the protein incapable of forming the inactive conformation.⁶² *JAK2* exon 12 mutations have been found in 3% of patients with PV and are not associated with ET or PMF but can be found in patients who progress to secondary myelofibrosis.^{74,75}

Experts hypothesize that mutations in signaling molecules alone are insufficient to initiate MPNs, suggesting that other mutations are necessary prior to *JAK2* V617F to induce disease and later to drive progression. Four lines of evidence support this hypothesis: familial MPN expresses a classic PV or ET phenotype in the absence of *JAK2* V617F or *MPL* W515L mutations and transmits in an autosomal dominant fashion; in some ET and PV clones that were erythropoietin independent, *JAK2* V617F was identified in a minority of cells, indicating that a pre-*JAK2* mutation drove the disease; approximately 50% of patients who developed acute leukemia from a *JAK2* V617F form of MPN expressed wild-type *JAK2*, suggesting a line of clonal evolution independent of *JAK2*; and in patients with PV and ET at diagnosis, the *JAK2* V617F allele burden in HSCs was low compared to the allele burden in later stages of hematopoiesis, suggesting that *JAK2* V617F confers a weak proliferative advantage to HSCs.⁶²

Three reports in 2010 identified a germline haplotype block that predisposes patients to *JAK2* mutations.⁷⁶⁻⁷⁸ This haplotype block was identified as a single nucleotide polymorphism (rs10974944) located in intron 12 of the *JAK2* gene, increasing the development of MPN by three- to fourfold.⁶¹

Also in 2010, mutations were discovered in the adapter protein LNK (aka Src homology 2 B3-SH2B3), which down-regulates JAK-STAT signaling pathways by regulating *JAK2* activation. Approximately 3% to 6% of patients with MPN bear an LNK mutation,^{79,80} with approximately 13% of mutations appearing in the blast phase versus the chronic phase of the disease.⁸¹ Following the binding of the corresponding ligand to its receptor, LNK binds to erythropoietin receptor

(EPO-R), thrombopoietin receptor (*MPL*), and *JAK2* to down-regulate the JAK-STAT pathway as a negative modulator. Mutations in *LNK* produce a loss of function that removes a level of inhibitory control, increasing the proliferation of erythrocytes and thrombocytes. This loss of function mutation is accentuated in the presence of *JAK2* V617F and *MPL* W515L mutations, resulting in the PV and ET phenotypes, respectively.⁶¹ More recently, somatic mutations have been identified in genes that control DNA methylation in patients with PV and other MPNs. The most notable are *TET2*, *IDH1*, and *IDH2*. *TET2* (Ten Eleven Translocation 2) is one of three members of the *TET* family of genes (*TET1* and *TET3*) and the only one identified with sequence alterations. *TET2* appears to be highly mutagenic for three reasons: mutations have been identified in all types of myeloid disorders to include MPNs, MDSs, and AMLs; mutations have been found in all coding regions of the gene; and mutations are often biallelic (homozygous).⁸² *TET2* catalyzes the reaction that oxidizes the 5-methyl group of cytosine (5-mC) to 5-hydroxymethylcytosine (5-hmC).⁸³ It is hypothesized that 5-hmC serves as an intermediate base in the demethylation of DNA. Methylation of histones serves to silence genes. Therefore, *TET2* mutations produce a loss of function effect, resulting in hypermethylation and a loss of gene activation (inactivation of tumor suppressor genes).⁶¹ In addition, it appears that *TET2* mutations precede *JAK2* mutations based on three observations: *TET2* mutations are expressed in CD34+ hematopoietic stem cells (HSC); *TET2* mutations have been identified in all forms of myeloid disorders; and all patients with both *TET2* and *JAK2* mutations produced clones with both mutations and clones that were *TET2* positive and *JAK2* negative but none that were *JAK2* positive and *TET2* negative. Therefore, *TET2* mutations may create abnormal clones that predispose to *JAK2* mutations.⁸² *TET2* mutations have been identified in 9.8% to 16% of PV, 4.4% to 5% of ET, and 7.7% to 17% of PMF patients.⁶¹

Mutations in the genes that code for the citric acid cycle enzymes isocitrate dehydrogenase 1 (*IDH1*) and isocitrate dehydrogenase 2 (*IDH2*) have been associated with hypermethylation of DNA in patients with MPN and AML.⁸⁴ Normally these enzymes function to convert isocitrate to α -ketoglutarate, requiring the reduction of NAD(P)⁺ to NADPH as the energy source. In patients with MPNs, mutations in *IDH1/2* occur most frequently at the *IDH2* R140 residue, the *IDH1* R132 residue, and the *IDA2* R172 residue.⁸⁵ These mutations are thought to alter enzyme function, causing the conversion of α -ketoglutarate to 2-hydroxyglutarate (2-HG).⁸⁶ It is hypothesized that because *TET2* is dependent on α -ketoglutarate, the *IDH1/2* mutations would result in less α -ketoglutarate, thus impairing the function of *TET2* and exacerbating the hypermethylation function of mutated *TET2* protein products.⁸⁴ *IDH1/2* mutations occur most frequently (21.6% of patients) in late-stage PV and ET patients during blast transformation compared to an incidence of 1% to 5% in patients with early PV (1.9%), ET (0.8%), and PMF (4.2%).⁶¹ As expected, *IDH1/2* mutations are associated with adverse overall survival, raising the potential of using *IDH1/2* mutational analysis and/or 2-HG detection as poor prognostic indicators.

Mutations in two additional genes involved in epigenetic modification, *EZH2* and *ASXL1*, have been implicated in MPN, MDS, and MDS/MPN and, along with *TET2*, may precede *JAK2* V617F mutations. *EZH2* and *EZH1* function as two of several proteins that form the polychrome repressive complex 2 (PRC2) that regulates chromatin structure. More specifically, *EZH1* and *EZH2* provide the functional domain for the PRC2 complex to methylate histone H3 at lysine 27.⁸⁷ The array of mutations noted in *EZH2* to date function to either eliminate protein production or abrogate methyltransferase activity. Mutations in *EZH2* have been identified in 3% of PV patients, 13% of PMF patients, 12.3% of MDS/MPN patients, and 5.8% to 23% of MDS patients.^{88,89} *ASXL1* (Additional Sex Combs-like 1) mutations have been identified in most myeloid malignancies and at a frequency similar to *TET2*.⁶² *ASXL1* normally functions in conjunction with *ASXL2* and *ASXL3* to deubiquitinate histone H2 to balance the activity of PRC1 to monoubiquitinate target genes to modify chromatin structure.^{90,91} Ubiquitination tags proteins for natural removal, thus regulating their function. The function of *ASXL1* in hematopoiesis is poorly understood, but the loss of function mutations have been identified in less than 7% of PV and ET and from 19% to 40% in PMF.^{92,93}

Disease progression to blast crisis occurs in less than 10% of PV and ET patients, but several genetic mutations are implicated in this transformation.⁹⁴⁻⁹⁶ In addition to those that modulate epigenetic changes previously discussed (*IDH1/2* and *TET2*), mutations in *TP53* and *RUNX1* are involved in blast transformation. *TP53* produces the P53 protein that is known to be a tumor suppressor gene. P53 controls cell cycle checkpoints and apoptosis, and loss-of-function mutations are implicated in a host of cancers to include disease progression in the classic MPNs. *TP53* mutations have not been identified in MPNs in the chronic phase but have been found in 20% of patients with MPNs who have progressed to AML.⁹⁷⁻⁹⁹ The protein product of the *RUNX1/AML1* gene is a transcription factor that is important in hematopoiesis. *RUNX1* mutations were observed in 30% of post-MPN-AML patients, making it a candidate for the most frequent mutation involved in MPN transformation to AML.⁶²

Diagnosis

Based on the WHO standards, the diagnosis of PV requires that two major criteria and one minor criterion be met or that the first major criterion listed and two minor criteria be met. The two major criteria are an elevated hemoglobin (Hb) level (>18.5 g/dL in men and >16.5 g/dL in women) and the identification of the *JAK2* V617F mutation, the *JAK2* exon 12 mutation, or a similar *JAK2* mutation. The three minor criteria are panmyelosis in the bone marrow; low serum erythropoietin levels; and autonomous, in vitro erythroid colony formation.⁸ Additional diagnostic features of PV include an increased RBC mass of 36 mL/kg or greater in males and 32 mL/kg or greater in females, an arterial oxygen saturation of 92% (normal) or greater, and splenomegaly. Other features of PV are thrombocytosis of greater than 400×10^9 platelets/L; leukocytosis of greater than 12×10^9 cells/L without fever or infection; and increases in leukocyte alkaline phosphatase (LAP), serum

vitamin B₁₂, or unbound vitamin B₁₂ binding capacity.^{100,101} Recent research indicates that the *JAK2* V617F mutation can be expected in more than 90% to 95% of cases.⁸ The WHO criteria for the diagnosis of PV are summarized in [Box 33-1](#).

It is not always easy to assign an early diagnosis of PV. Erythrocytosis secondary to hypoxia or erythropoietin-producing neoplasms are the most difficult to diagnose correctly. In individuals with these conditions, the bone marrow exhibits erythroid hyperplasia without granulocytic or megakaryocytic hyperplasia. Patients with stress or spurious erythrocytosis exhibit increased hemoglobin and hematocrit (HCT) without increased erythrocyte mass or splenomegaly.

Peripheral Blood and Bone Marrow

Common peripheral blood, bone marrow, and tissue findings in the early or proliferative phase of PV are listed in [Table 33-2](#). [Figures 33-11](#) and [33-12](#) show common morphologic patterns in peripheral blood and bone marrow morphologic and cellular changes. Not only are quantitative changes seen, but bone marrow normoblasts may collect in large clusters, megakaryocytes are enlarged and exhibit lobulated nuclei, and bone marrow sinuses are enlarged without fibrosis. Pseudo-Gaucher cells are rare.²⁸ Approximately 80% of patients manifest bone marrow panmyelosis, and 100% of bone marrow volume may exhibit hematopoietic cellularity. Although the bone marrow pattern may mimic that of other MPNs, the peripheral blood cells appear normal, with normocytic, normochromic erythrocytes; mature granulocytes; and normal-sized, granulated

BOX 33-1 World Health Organization Criteria for the Diagnosis of Polycythemia Vera

Diagnosis requires the presence of both major criteria and one minor criterion or the presence of the first major criterion together with two minor criteria.

Major Criteria

1. Hemoglobin >18.5 g/dL in men, >16.5 g/dL in women or other evidence of increased red blood cell volume*
2. Presence of *JAK2* V617F or other functionally similar mutation such as *JAK2* exon 12 mutation

Minor Criteria

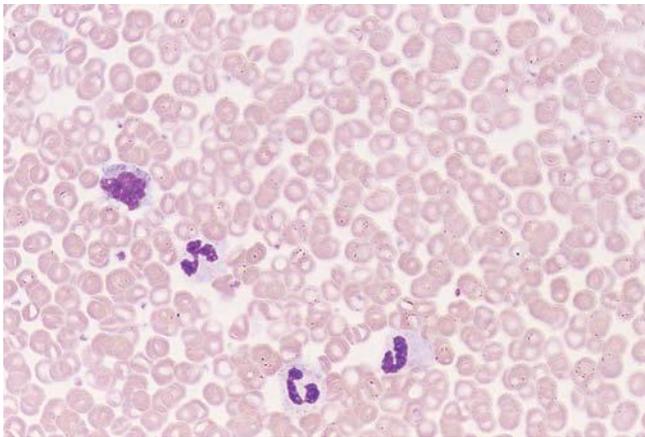
1. Bone marrow biopsy specimen showing hypercellularity for age with trilineage growth (panmyelosis) with prominent erythroid, granulocytic, and megakaryocytic proliferation
2. Serum erythropoietin level below the reference range for normal
3. Endogenous erythroid colony formation in vitro

*Hemoglobin or hematocrit >99th percentile of method-specific reference range for age, sex, altitude, or residence, or hemoglobin >17 g/dL in men, >15 g/dL in women if associated with a documented and sustained increase of at least 2 g/dL from an individual's baseline value that cannot be attributed to correction of iron deficiency, or elevated red cell mass >25% above mean normal predicted value.

From Vardiman JW, Thiele J, Arber DA, et al: The 2008 revision of the World Health Organization (WHO) classification of myeloid neoplasms and acute leukemia: rationale and important changes. *Blood* 114:937-951, 2009.

TABLE 33-2 Common Morphologic Changes in Polycythemia Vera

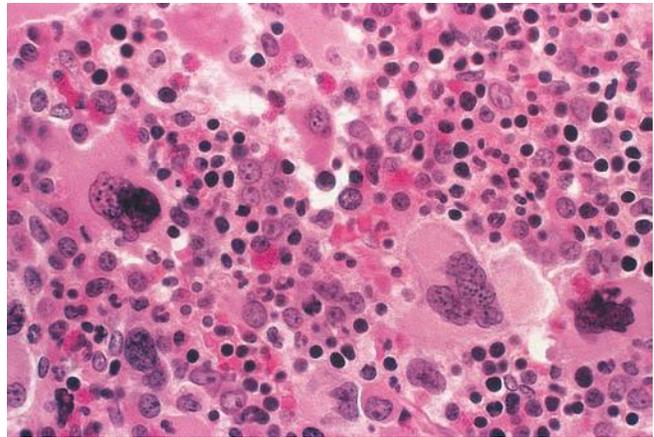
Peripheral Blood	
Hemoglobin	Increased
Hematocrit	Increased
Red blood cell volume	Increased
Erythrocyte morphology	Normocytic/Normochromic
Total white blood cells	Increased
Granulocytes	Increased
Platelets	Increased
Leukocyte alkaline phosphatase	Normal or increased
Bone Marrow	
Normoblasts	Increased
Granulocytes	Increased
Megakaryocytes	Increased
Reticulin	Increased
Extramedullary Tissue	
Splenomegaly	Present
Sinusoidal	Present
Medullary	Present
Hepatomegaly	Present
Sinusoidal	Present

**Figure 33-11** Peripheral blood film in stable phase polycythemia vera with essentially normocytic, normochromic erythrocytes ($\times 500$).

platelets. The other 20% of patients exhibit lesser degrees of cellularity in the bone marrow and peripheral blood. Splenomegaly, hepatomegaly, generalized vascular engorgement, and circulatory disturbances increase the risk of hemorrhage, tissue infarction, and thrombosis.

Clinical Presentation

PV initially manifests in a proliferative phase independent of normal regulatory mechanisms. PV is always associated with increased RBC mass. This is the stable phase of PV, which progresses to a spent phase in a few patients. In the spent phase, patients experience progressive splenomegaly (palpable

**Figure 33-12** Bone marrow biopsy specimen in stable phase polycythemia vera showing panmyelosis (hematoxylin and eosin stain, $\times 400$).

spleen) or hypersplenism (large spleen with bone marrow hyperplasia and peripheral blood cytopenias) and pancytopenia. They may also exhibit the triad of bone marrow fibrosis, splenomegaly, and anemia with teardrop-shaped poikilocytes. The latter pattern is called *postpolycythemic myeloid metaplasia*, and its morphologic features are similar to those of PMF. Peripheral WBC and RBC counts vary, and nucleated erythrocytes, immature granulocytes, and large platelets are present. Usually, splenomegaly is secondary to extramedullary hematopoiesis.¹⁰² Myelofibrosis occurs within the bone marrow and may come to occupy a significant proportion of bone marrow volume, with subsequent ineffective hematopoiesis.¹⁰³

Treatment and Prognosis

The treatment of choice for PV is therapeutic phlebotomy at a frequency necessary to maintain the hematocrit at less than 45%. Low-dose aspirin has been shown efficacious to minimize thrombosis in all risk categories.¹⁰⁴ The alkylating agent hydroxyuria is recommended in high-risk patients with PV and can be substituted for INF- γ in younger patients^{105, 106} and busulfan in older patients who develop intolerance or resistance to hydroxyuria.¹⁰⁷ Prognosis for patients with PV is good, with a median survival exceeding 15 to 20 years.¹⁰⁸ However, the disease progresses to acute leukemia in 15% of patients. The use of myelosuppressive therapy such as phosphorus P 32 (³²P) or alkylating agents seems to increase the risk.¹⁰¹ Only 1% to 2% of patients treated with phlebotomy alone experience leukemic transformation. However, the risk of thrombosis and bleeding is increased in patients treated with phlebotomy alone, so the use of alkylating myelosuppressive agents may be required to control these complications. Some patients may manifest a temporary disease pattern similar to myelodysplasia, and the cell morphology in transformation to acute leukemia may be difficult to classify. Patients with both early and advanced PV may show clinical, peripheral blood, bone marrow, and extramedullary features that mimic those of other MPNs.

Treatment with modern JAK inhibitors has provided important benefits to patients with PV. A total of 34 patients intolerant or refractory to hydroxyuria were enrolled in the phase II study and treated with INCB018424 (ruxolitinib) at a dose of

10 mg b.i.d. Of the 34 patients, 15 (45%) had a complete remission.¹⁰⁹ In addition, 97% (32 patients) achieved phlebotomy independence, and 80% (27 patients) achieved a 50% decrease in spleen size, as well as a reduction in pruritus, bone pain, and night sweats.¹¹⁰ CEP-701 (Lestaurtinib) was studied on 27 PV and 12 ET patients refractory or intolerant to hydroxyuria, and the outcomes were less promising. In 15 of the 39 patients who completed 18 weeks of treatment, 83% (15/18) achieved some degree of spleen size reduction, 60% (3/5) had a reduction in phlebotomy requirements, 20% (3/15) had a 15% decrease in *JAK2* allele burden, and 15% developed thrombosis; gastrointestinal events were frequent.¹¹¹

ESSENTIAL THROMBOCYTHEMIA

Essential thrombocythemia (ET) is a clonal MPN with increased megakaryopoiesis and thrombocytosis, usually with a count greater than $600 \times 10^9/L$ and sometimes with a count greater than $1000 \times 10^9/L$.¹¹² However, WHO criteria require a sustained thrombocytosis with a platelet count of $450 \times 10^9/L$ or greater. Over the years, ET has been known as *primary thrombocytosis*, *idiopathic thrombocytosis*, and *hemorrhagic thrombocythaemia*.^{3,113}

Incidence

In the absence of a well-defined diagnostic algorithm, determining the true incidence of ET has been difficult. When the diagnostic system developed by the Polycythemia Vera Study Group (PVSG) is applied, however, the incidence is estimated to be between 0.6 and 2.5 cases per 100,000 persons per year. The majority of cases occur in individuals between the ages of 50 and 60 years, but a second peak occurs primarily in women in the childbearing years, approximately 30 years of age.¹¹³

Pathogenic Mechanism

Most of the mutations described in PV also occur in ET but usually at a lower frequency. The *JAK2* V617F occurs in approximately 55% of patients with ET.¹¹⁴ *MPL* exon 10 mutations (*MPL* W515L/K) are observed in 3% of ET patients, as well as several other mutations previously discussed to include *TET2* (4.4% to 5%), *ASXL1* (5.6%), *LNK* (3% to 6%), and *IDH1/2* (0.8%).⁶¹ The manner in which these mutations alter normal cellular functions is similar to PV, as previously described.

Clinical Presentation

In more than one half of the patients diagnosed with ET, the disorder is discovered in the laboratory by virtue of an unexpectedly elevated platelet count on a routine complete blood count; the remaining patients see a physician due to vascular occlusion or hemorrhage. Vascular occlusions are often the result of microvascular thromboses in the digits or thromboses in major arteries and veins that occur in a variety of organ systems, including splenic or hepatic veins, as in Budd-Chiari syndrome. Bleeding occurs most frequently from mucous membranes in the gastrointestinal and upper respiratory tracts. Splenomegaly is observed at presentation in 50% of patients when the PVSG

diagnostic criteria are used but at a much lower frequency when the WHO standards are applied. This difference is largely due to the elimination of the diagnosis of ET in patients who meet the criteria for PMF in the prefibrotic stage.¹¹³

Diagnosis

ET must be differentiated from secondary or reactive thrombocytoses and from other MPNs. Thrombocytosis may be secondary to chronic active blood loss, hemolytic anemia, chronic inflammation or infection, or nonhematogenous neoplasia. The diagnostic criteria for ET first proposed by the PVSG were intended to distinguish ET from other MPNs. These features included a platelet count of greater than $600 \times 10^9/L$, a hemoglobin of less than 13 g/dL or a normal erythrocyte mass, and stainable iron in the bone marrow or a failure of iron therapy. Philadelphia chromosome negativity, absence of marrow collagen fibrosis (less than one third of a biopsy specimen is fibrous), no splenomegaly, absence of leukoerythroblastic reaction, and no known cause of reactive thrombocytosis all support the diagnosis.¹¹⁵

The newest WHO group now requires the documentation of four major criteria to establish a diagnosis of ET. First, the WHO group lowered the platelet count threshold to $450 \times 10^9/L$ or greater to capture patients who would eventually meet diagnostic criteria but who were experiencing hemorrhage or thrombosis with platelet counts of between $450 \times 10^9/L$ and $600 \times 10^9/L$.¹¹³ Because a lower platelet threshold could lead to false-positive ET diagnoses, all the WHO criteria must be met to eliminate such patients. Second, the bone marrow must show significant megakaryopoiesis characterized by large, mature-looking megakaryocytes with no substantial increase in erythropoiesis or granulopoiesis or left shift in the neutrophil line. Third, the condition cannot meet the criteria of any other MPN, myelodysplasia, or other myeloid neoplasm. Fourth, patients must demonstrate either the *JAK2* V617F or other clonal mutation or, in the absence of a clonal marker, the absence of reactive thrombocytosis. Careful analysis of the bone marrow biopsy specimen is useful in distinguishing ET from myelodysplastic syndromes (MDSs) associated with the del(5q) mutation, refractory anemia with ringed sideroblasts with thrombocytosis, and the prefibrotic phase of PMF. Likewise, the identification of the *JAK2* V617F mutation excludes cases of reactive thrombocytosis.¹¹³

The *JAK2* V617F mutation is found in 50% to 60% of ET patients and supports the diagnosis of ET.^{116,117} WHO diagnostic criteria were modified to include a minimum platelet count of $450 \times 10^9/L$ when the *JAK2* mutation is present.¹¹⁸ *JAK2* mutations have not been identified in the germline of any patient with MPN disorder, which supports the view that the mutation is acquired. *MPL* W515K/L, a mutation in the thrombopoietin receptor (*MPL*), has been reported in 3% of ET cases and is also used to exclude a diagnosis of reactive thrombocytosis.¹¹⁴ Other genetic mutations are uncommon but have been reported to be found in 5% to 10% of cases when the diagnostic criteria proposed by the PVSG are applied. The most commonly reported additional mutations are +8, 9q, and (del)20q.¹¹³

Peripheral Blood and Bone Marrow

Figure 33-13 shows a peripheral blood film that exhibits early-phase thrombocytosis with variation in platelet diameter and shape, including giantism, agranularity, and pseudopods. Commonly, platelets are present in clusters and tend to accumulate near the thin edge of the blood film. Segmented neutrophils may be increased; basophils are not. Erythrocytes are normocytic and normochromic, unless iron deficiency is present secondary to excessive bleeding.

Early-phase bone marrow shows marked megakaryocytic hypercellularity, clustering of megakaryocytes, and increased megakaryocyte diameter with nuclear hyperlobulation and density (Figure 33-14). Special studies reveal increased numbers of smaller and less mature megakaryocytes.¹¹⁹ Increased granulopoiesis and erythropoiesis may contribute to bone marrow hypercellularity, and, in a few patients, reticulin fibers may be increased. The major peripheral blood, bone marrow, and extramedullary findings are listed in Table 33-3.

A diagnosis of ET is questionable in patients with a platelet count of more than $450 \times 10^9/L$ if certain features are observed

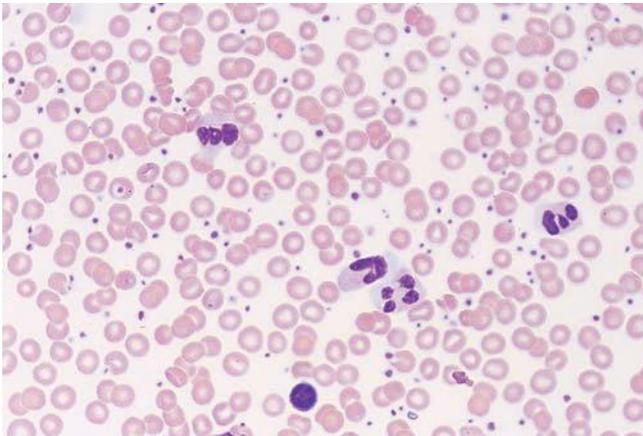


Figure 33-13 Peripheral blood film in stable phase essential thrombocythemia showing increased numbers of platelets and mature neutrophils ($\times 500$).

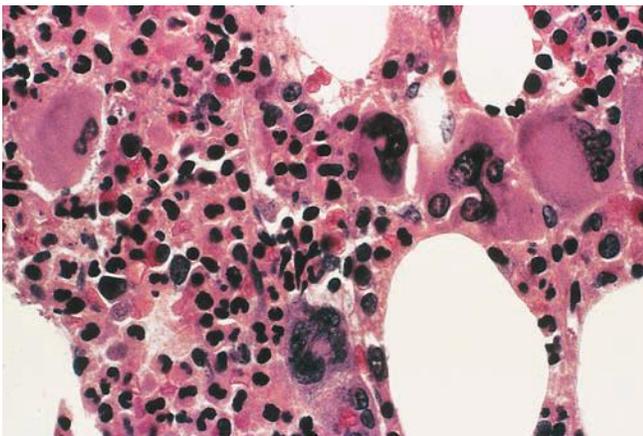


Figure 33-14 Bone marrow biopsy specimen in essential thrombocythemia showing marked megakaryocytic hypercellularity (hematoxylin and eosin stain, $\times 400$).

TABLE 33-3 Common Morphologic Changes in Essential Thrombocythemia

Peripheral Blood	
Hemoglobin	Slightly decreased
Hematocrit	Slightly decreased
Red blood cell volume	Normal
Total white blood cells	Normal or slightly increased
Neutrophils	Normal or slightly increased
Platelets	Increased
Platelet function	Decreased
Bone Marrow	
Normoblasts	Normal or increased
Granulocytes	Normal or slightly increased
Megakaryocytes	
Clusters	Present
Large	Present
Hyperlobulated	Present
Dense nuclei	Present
Variability in size	Increased
Reticulin	Normal or slightly increased
Extramedullary Tissue	
Splenomegaly	Present
Sinusoidal	Present
Medullary	Present
Megakaryocytic proliferation	Present

on the bone marrow biopsy specimen. For example, increased erythropoiesis or granulopoiesis in the bone marrow is a questionable finding for ET and suggests an alternative diagnosis of PV or PMF, respectively, especially if bizarre or significantly atypical megakaryocytes are also observed. Dyserythropoiesis and/or dysgranulopoiesis suggests a myelodysplastic disorder and should prompt an investigation for (del)5q, (inv)3, and/or t(3;3).¹¹³

Treatment and Prognosis

Treatment involves prevention or early alleviation of hemorrhagic or vasoocclusive complications that occur as the platelet count increases. The production of platelets must be reduced by suppressing marrow megakaryocyte production with an alkylating agent like hydroxyurea. As observed in PV, ET patients so treated may incur an increased risk for disease transformation to acute leukemia or myelofibrosis. However, malignant transformation occurs at a frequency of less than 5%.¹¹⁴ Hydroxyurea therapy may achieve a desired reduction of peripheral platelets without the risk of complications experienced with myelosuppressive agents. This may relate to the youth of ET patients, in whom the risk of leukemic transformation seems relatively low. For patients who develop intolerance or resistance to hydroxyurea, cytoreduction can be achieved with interferon- α in younger patients¹⁰⁵ and busulfan in older patients.¹²⁰ Low-dose aspirin is also recommended to prevent thrombosis.¹¹⁴

JAK2 inhibitors are being investigated in ET patients who are refractory or intolerant to hydroxyurea or are otherwise

high risk. INCB018424 (ruxolitinib) was studied in 39 patients with ET at a dose of 25 mg b.i.d. In all 39 patients, the median platelet count reduced from 884 to $558 \times 10^9/L$, and the 11 patients who had leukocytosis achieved a normal WBC count after 6 months of treatment. Four patients who demonstrated splenomegaly showed spleen size reduction; 40% to 75% of patients had a 50% or greater improvement in one or more of the following: pruritus, bone pain, night sweats, and peripheral tingling/numbness. Only 13% (5 patients), achieved complete remission. However, a follow-up report at 10.4 months of treatment showed that 92% were still participating in the study, no grade 3 or 4 hematologic complications were noted, and although cytopenias were observed in 10% to 20%, they were grade 2 (mild).¹¹⁰ CEP701 (Lestaurtinib) was studied in 27 patients with PV and 12 patients with ET who were refractory or intolerant to hydroxyuria. In 15 of the 39 patients who completed 18 weeks of treatment, 83% (15/18) achieved some degree of spleen size reduction, 20% (3/15) had a 15% decrease in *JAK2* allele burden, and 15% developed thrombosis and had frequent gastrointestinal events.¹¹¹

Patients with ET experience relatively long survival provided they remain free of serious thromboembolic or hemorrhagic complications. Clinical symptoms associated with thromboembolic vasoocclusive events include the syndrome of erythromelalgia (throbbing and burning pain in the hands and feet, accompanied by mottled redness of areas), transient ischemic attacks, seizures, and cerebral or myocardial infarction. Other symptoms include headache, dizziness, visual disturbances, and dysesthesias (decreased sensations). Hemorrhagic complications include bleeding from oral and nasal mucous membranes or gastrointestinal mucosa and the appearance of cutaneous ecchymoses (Chapter 40).

The median survival for patients with ET is 20 years, including cases in which the process arises in younger patients.¹²¹ However, some patients may develop post-ET myelofibrosis, which reduces survival. Patients whose cells manifest chromosome abnormalities may have a poorer prognosis.³⁹

PRIMARY MYELOFIBROSIS

Primary myelofibrosis (PMF), previously known as *chronic idiopathic myelofibrosis*, *agnogenic myelofibrosis*, and *myelofibrosis with myeloid metaplasia*, is a clonal MPN⁶ in which there is splenomegaly and ineffective hematopoiesis associated with areas of marrow hypercellularity, fibrosis, and increased megakaryocytes. Megakaryocytes are enlarged with pleomorphic nuclei, coarse segmentation, and areas of hypochromia. The peripheral blood film exhibits immature granulocytes and normoblasts, dacryocytes (teardrop-shaped RBCs), and other bizarre RBC shapes.

PMF clonality was manifest in studies in which cytogenetic abnormalities were detected in normoblasts, neutrophils, macrophages, basophils, and megakaryocytes. Female patients heterozygous for glucose-6-phosphate dehydrogenase isoenzymes have PMF cells of a single enzyme isotype, whereas tissue cells, including marrow fibroblasts, contain both enzyme isotypes.⁶

Myelofibrosis

The myelofibrosis in this disease consists of three of the five types of collagen: I, III, and IV. Increases in type III collagen are detected by silver impregnation techniques, increases in type I by staining with trichrome, and increases in type IV by the presence of osteosclerosis, which may be diagnosed from increased radiographic bone density.¹²² In approximately 30% of patients, biopsy specimens show no fibrosis.³⁹ Increases in these collagens are not a part of the clonal proliferative process but are considered secondary to an increased release of fibroblastic growth factors, such as platelet-derived growth factor, transforming growth factor α from megakaryocyte α -granules, tumor necrosis factor- α , and interleukin-1 α and interleukin-1 β . Marrow fibrosis causes expansion of marrow sinuses and vascular volume, with an increased rate of blood flow. Bone marrow fibrosis is not the sole criterion for the diagnosis of PMF because increases in marrow fibrosis may reflect a reparative response to injury from benzene or ionizing radiation, may be a consequence of immunologically mediated injury, or may represent a reactive response to other hematologic conditions.

Type IV collagen and laminin normally are discontinuous in sinusoidal membranes but appear as stromal sheets in association with neovascularization and endothelial cell proliferation in regions of fibrosis. In addition, deposition of type VII collagen is observed, and this may form a linkage between type I fibers, type III fibers, and type I plus type III fibers.¹²³

Hematopoiesis and Extramedullary Hematopoiesis

Extramedullary hematopoiesis, clinically recognized as hepatomegaly or splenomegaly, seems to originate from release of clonal stem cells into the circulation.¹²⁴ The cells accumulate in the spleen, liver, or other organs, including adrenals, kidneys, lymph nodes, bowel, breasts, lungs, mediastinum, mesentery, skin, synovium, thymus, and lower urinary tract. The cause of extramedullary hematopoiesis is unknown. In experimental animal models, chemicals, hormones, viruses, radiation, and immunologic factors have been implicated. The disease is associated with an increase in circulating hematopoietic cells, but fibroblasts are a secondary abnormality and not clonal.¹²⁵ B and T cells may be involved.¹²⁶ There is an increase in circulating unilineage and multilineage hematopoietic progenitor cells,¹²⁷ and the number of CD34⁺ cells may be 300 times normal.¹²⁸ The increase in circulating CD34⁺ cells separates PMF from other MPNs and predicts the degree of splenic involvement and risk of conversion to acute leukemia.

Body cavity effusions containing hematopoietic cells may arise from extramedullary hematopoiesis in the cranium, the intraspinal epidural space, or the serosal surfaces of pleura, pericardium, and peritoneum. Portal hypertension, with its attendant consequences of ascites, esophageal and gastric varices, gastrointestinal hemorrhage, and hepatic encephalopathy, arises from the combination of a massive increase in splenoportal blood flow and a decrease in hepatic vascular

compliance secondary to fibrosis around the sinusoids and hematopoietic cells within the sinusoids.¹²⁹

Pathogenetic Mechanism

As with PV and ET, the *JAK2* V617F mutation is involved in the pathogenesis and is found in 65% of PMF patients.¹¹⁴ The *MPL* W515L/K occurs in an additional 10% of patients, along with most of the other mutations previously discussed, to include *CBL* (6%),¹³⁰ *TET2* (7.7% to 17%), *ASXL1* (13% to 23%), *LNK* (3% to 6%), *EZH2* (13%), and *IDH1/2* (4.2%).⁶¹

Incidence and Clinical Presentation

The disease occurs in patients older than age 60 and may be asymptomatic. PMF generally presents with fatigue, weakness, shortness of breath, palpitations, weight loss, and discomfort or pain in the left upper quadrant associated with splenomegaly.

Peripheral Blood and Bone Marrow

PMF presents with a broad range of changes in laboratory test values and peripheral blood film results, but examination of the bone marrow biopsy specimen provides most of the information for diagnosis. Changes commonly observed in peripheral blood and bone marrow examinations are summarized in Table 33-4.

Abnormalities in erythrocytes noted on peripheral blood films include the presence of dacryocytes, other bizarre shapes, nucleated RBCs, and polychromatophilia. Granulocytes are increased, normal, or decreased in number and may include immature granulocytes, blasts, and cells with nuclear or cytoplasmic anomalies. Platelets may be normal, increased, or decreased in number, with a mixture of normal and abnormal morphologic features (Figure 33-15). Micromegakaryocytes may be observed (Figure 33-16).

Bone marrow biopsy specimens exhibit intense fibrosis, granulocytic and megakaryocytic hypercellularity, dysmegakaryopoiesis, dysgranulopoiesis, and numerous dilated sinuses containing luminal hematopoiesis. Neutrophils may exhibit impairment of physiologic functions such as phagocytosis, oxygen consumption, and hydrogen peroxide generation, and decreased myeloperoxidase and glutathione reductase activities. Platelets show impaired aggregation in response to epinephrine, decreased adenosine diphosphate concentration in dense granules, and decreased activity of platelet lipooxygenase.

Immune Response

Humoral immune responses are altered in approximately 50% of patients and include the appearance of autoantibodies to erythrocyte antigens, nuclear proteins, gamma globulins, phospholipids, and organ-specific antigens.¹³¹ Circulating immune complexes, increased proportions of marrow-reactive lymphocytes, and the development of amyloidosis are evidence for active immune processes. Collagen disorders coexist with PMF, which suggests that immunologic processes may stimulate marrow fibroblast activity.

Treatment and Prognosis

A diverse spectrum of therapies has been implemented to alleviate symptoms or modify clinical problems in patients with

TABLE 33-4 Common Morphologic Changes in Primary Myelofibrosis

Peripheral Blood	
Hemoglobin	Normal or decreased
Anisocytosis	Present
Poikilocytosis	Present
Teardrop-shaped erythrocytes	Present
Nucleated red blood cells	Present
Polychromasia	Normal or increased
Total white blood cells	Normal, decreased, or increased
Immature granulocytes	Increased
Blasts	Present
Basophils	Present
Leukocyte anomaly	Present
Leukocyte alkaline phosphatase	Increased, normal, or decreased
Platelets	Increased, normal, or decreased
Abnormal platelets	Present
Megakaryocytes	Present
Bone Marrow	
Cellularity	Increased
Granulopoiesis	Increased
Megakaryocytes	Increased
Erythropoiesis	Normal or increased
Myelofibrosis	Increased
Sinuses	Increased
Dysmegakaryopoiesis	Present
Dysgranulopoiesis	Present
Extramedullary Tissue	
Splenomegaly	Present
Sinusoidal	Present
Medullary	Present
Hepatomegaly	Present
Sinusoidal	Present
Portal tract	Present
Local infiltrates	Present
Other tissues	Present

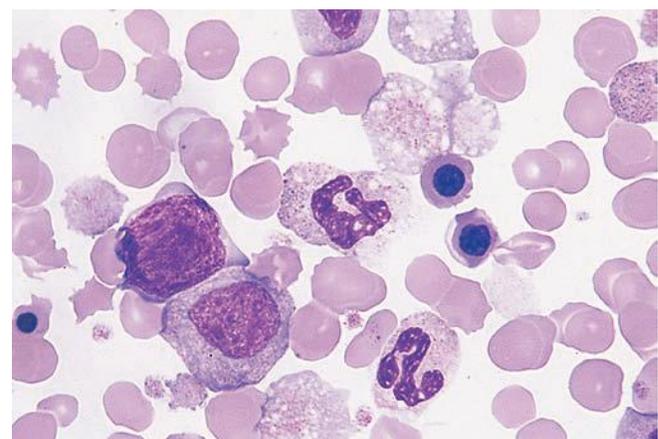


Figure 33-15 Peripheral blood film in primary myelofibrosis showing nucleated red blood cells, giant platelets, and immature myeloid cells ($\times 1000$).

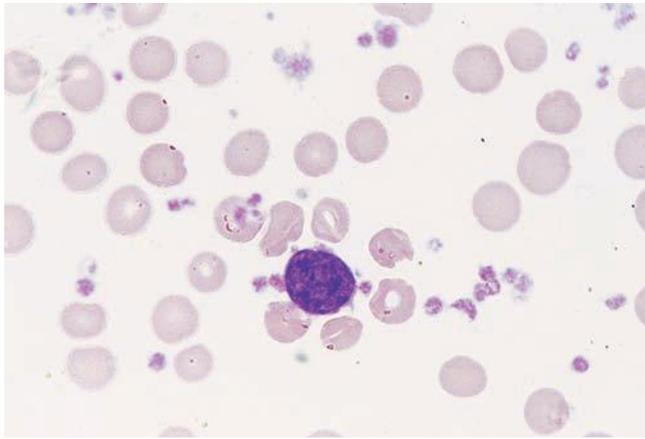


Figure 33-16 Peripheral blood film in primary myelofibrosis exhibiting increased platelets and a micromegakaryocyte ($\times 1000$).

PMF, but none has been disease-modifying, so treatment approaches have been largely palliative. Treatment has been targeted at the amelioration of anemia, hepatosplenomegaly, and constitutional symptoms. Between 34% and 54% of PMF patients present with a hemoglobin of less than 10 g/dL.^{132,133} Severe anemia has been treated with androgen therapy, prednisone, danazol,¹³⁴ thalidomide,^{135,136} or lenalidomide,¹³⁷ and hemolytic anemia with glucocorticosteroids. Approximately 20% of patients respond with an average duration of 1 to 2 years. Thalidomide and lenalidomide must be used with caution due to the occurrence of neuropathies and myelosuppression, particularly if the patient has been identified with del(5q31).¹³⁸

Splenomegaly is present in 90% of patients with PMF, and 50% show hepatomegaly.^{132,133} The most common first-line therapy for splenomegaly is hydroxyurea, but caution must be exercised so as not to exacerbate preexisting cytopenias.^{139,140} Splenectomy and local radiation to the spleen and liver have been used in patients refractory to hydroxyurea, but patients must be carefully monitored for postoperative thrombosis, bleeding, infections, and cytopenias.¹⁴¹

The most common constitutional symptoms encountered by patients with PMF include fatigue (84%), bone pain (47%), night sweats (56%), pruritus (50%), and fever (18%).^{142,143} However, treatment to alleviate these symptoms are minimally effective.

The development and testing of JAK inhibitors were directed at PMF because the symptoms and outcomes are worse compared to those of PV and ET. Among the JAK2 inhibitors tested, the four that showed the most promise were two JAK1/2 inhibitors, INCB018424 (ruxolitinib) and CYT387; one JAK2 inhibitor, TG101348; and one non-JAK inhibitor, CEP-701 (Lestaurtinib).

INCB018424 (ruxolitinib) was evaluated in a phase I/II trial at a dose of 25 mg b.i.d. and 100 mg q.d. on 153 patients with high- and moderate-risk PMF, both with primary disease and those who progressed from PV and ET. Clinical improvement associated with splenomegaly reduction (smaller spleen without progressive disease or increase in severity of anemia, thrombocytopenia, or neutropenia) was reported in 44% (16/140), and 14% (4/28) who were transfusion dependent showed anemia

improvement, while 25% (38/153) withdrew from the study due largely to grade 3 thrombocytopenia and anemia (27%) that reduced to 16% following dose reduction to 10 mg b.i.d.¹⁴⁴ A majority of patients reported a 50% improvement in symptoms, probably due to a measurable reduction in proinflammatory cytokines. However, there was no appreciable reduction in JAK2 V617F allele burden.^{114,143} A phase III trial (COMFORT-1) of INCB018424 (ruxolitinib) is under way.

A phase I trial of TG101348 was conducted on 59 patients with high- and moderate-risk PMF at a maximum tolerated dose (MTD) of 680 mg q.d. Low-grade, transient nausea; vomiting; and diarrhea occurred in up to 69% of subjects, and along with adverse events, 45% withdrew from the study. Increases in serum lipase, transaminases, and creatinine occurred in approximately 25% of patients without any associated symptoms. Over 50% of subjects reported improvement in early satiety, night sweats, fatigue, pruritus, and cough along with a greater than 50% reduction in spleen size among 68% of the subjects. Thrombocytosis was normalized in 90% of patients and leukocytosis was normalized in 57%. In addition, 78% of patients had a 50% reduction in JAK2 V617F burden, while 13% increased and 9% remained unchanged.¹⁴⁵

CYT387 is in phase I trials using 36 patients with high- and moderate-risk PMF, including 28% who were previously treated with INCB018424 or TG101348. At an MTD of 300 mg/day, all subjects continued after 15 weeks of treatment. Drug-related thrombocytopenia was observed in 22% (grade 3 or 4), anemia in 3% (grade 3), first-dose effect of lightheadedness and hypotension in 36% (grade 1), and several incidences of grade 3 adverse events, including elevations in liver and pancreatic enzymes, headaches, and QTc prolongations on ECG tracings. Reduction in splenomegaly was observed in 97% of subjects, with 37% achieving a greater than 50% response. The majority of patients reported a reduction in fatigue, pruritus, night sweats, cough, bone pain, and fever, and 41% of patients with pretreatment anemia showed clinical improvement.¹⁴⁶

CEP-701 (lestaurtinib) was evaluated in phase II clinical trials at a dose of 80 mg b.i.d. using 20 patients who were JAK2 V617F positive. Twenty patients (91%) withdrew from the study for a variety of reasons, including diarrhea (73%), nausea (50%), vomiting (27%), headache (32%), mucositis (14%), peripheral neuropathy (14%), elevated transaminases (27%), anemia (27%), and thrombocytopenia (23%). Neither JAK2 V617F allele burden nor serum levels of proinflammatory cytokines were affected by therapy. However, 27% of subjects reported clinical improvement in anemia splenomegaly reduction.¹⁴⁷ A new phase I/II trial is under way with a new formulation of CEP-701 at higher doses.¹⁴⁸

Reduction of myelofibrosis and of marrow and tissue hypercellularity has been accomplished with busulfan hydroxyurea and, in a few patients, interferon- α and interferon- γ . Radiotherapy is considered for patients with severe splenic pain, patients with massive splenomegaly who are not clinical candidates for splenectomy, patients with ascites secondary to serosal implants (metastatic nodules), and patients with localized bone pain and localized extramedullary fibrohematopoietic masses in other areas, especially in the epidural space. Splenectomy is performed

to end severe pain, excessive transfusion requirements, or severe thrombocytopenia and to correct severe portal hypertension.

Chemotherapy is partially successful in reducing the number of CD34⁺ cells and immature hematopoietic cells, marrow fibrosis, and splenomegaly.¹⁴⁹ Single-agent chemotherapy is most helpful in the early clinical phases of the disease, and agents such as busulfan, 6-thioguanine, and chlorambucil, alone or in combination with other chemotherapy, are useful. Other therapies include interferon- α , hydroxyurea, and combinations of the previously mentioned drugs.¹⁵⁰ The most successful treatment to date for patients younger than age 60 is allogeneic stem cell transplantation. Five-year survival approaches 50% in patients undergoing transplantation, but 1-year mortality is 27%, and graft-versus-host disease occurs in 33%.¹⁵¹

Average survival from the time of diagnosis is about 5 years, but patients have lived as long as 15 years. During this time, increasing numbers and pleomorphy of megakaryocytes lead to progressive marrow failure. Marrow blasts may increase.³⁹ Adverse prognostic indicators include more severe anemia and thrombocytopenia, greater hepatomegaly, unexplained fever, and hemolysis. Mortality is associated with infection, hemorrhage, postsplenectomy complications, and transformation to acute leukemia.

SUMMARY OF CURRENT THERAPY OF NON-BCR/ABL1, PRIMARY MPNs

JAK2 inhibitors are most effective in patients with PMF due in part to the more severe symptoms in PMF compared to PV and ET. INCB018424, TG101348, and CYT387 appear to have a significant effect on decreasing splenomegaly—one of the more serious symptoms in PMF patients—within the first cycle of therapy, which peaks in 3 months. Splenic responses are dose dependent, durable through 12 treatment cycles, and limited by concomitant myelosuppression. Splenomegaly quickly returns with cessation of JAK2 inhibitors either within days for INCB018424 or within weeks for TG101348, due largely to their respective half-lives and possibly mode of action. The same three JAK2 inhibitors improve constitutional symptoms and appear to be durable. Treatment-related anemia is associated more with some JAK1/2 inhibitors (INCB018424) than others (CYT387) and is also associated with some JAK2 inhibitors (TG101348).¹¹⁴

In contrast, adverse events are dissimilar across the JAK inhibitors. For example, gastrointestinal events occur more frequently with the JAK2 inhibitor (TG101348) and with the non-JAK2 inhibitor (CEP-701), which might be due to the off-target FLT3 inhibition. Acute relapse of symptoms with drug discontinuation is seen in only one particular JAK1/2 inhibitor (INCB018424), which may be due to a “cytokine flare.” Lastly, only one of the JAK1/2 inhibitors (CYT387) produces first-dose symptoms of transient hypotension, flushing, and light-headedness. Future JAK inhibitor treatment may start with an induction dose to maximize response, followed by a maintenance dose with the addition and removal of other therapies tailored to the unique symptoms of each patient. For example, treatment-related myelosuppression could be ameliorated with

pomalidomide, androgens, erythropoietin, and transfusions, and constitutional symptoms can also be managed through a host of traditional therapies. Outcomes in PV and ET are not as impressive, and the need to modulate symptoms is not as critical. Nonetheless, the JAK2 inhibitor TG101348 is most useful due its ability to normalize leukocytosis and thrombocytosis.^{114,145}

INTERCONNECTION AMONG ESSENTIAL THROMBOCYTHEMIA, POLYCYTHEMIA VERA, AND PRIMARY MYELOFIBROSIS

The discovery of the JAK2 V617F mutation has advanced our understanding of the MPNs but has also raised questions about the interconnection of three of the primary myeloproliferative conditions: ET, PV, and PMF. Why is the JAK2 mutation found in more than 90% to 95% of patients with PV but in only 50% to 60% of patients with ET and PMF, and how can the same mutation produce three distinct phenotypes?

Currently four hypotheses exist to account for this apparent discordance. One prevailing thought suggests that the resulting phenotype is dependent on the stage of differentiation of the hematopoietic stem cell. For example, if the hematopoietic stem cell has developed a predilection toward platelet development at the time of the JAK2 mutation, ET will develop. Reports have described differences in differentiation programs^{152,153} and in JAK2 mutations among ET, PV, and PMF.¹⁵⁴ A second hypothesis proposes that the genetic background of the patient predisposes the patient to a particular phenotype. Mutations in the erythropoietin receptor, thrombopoietin receptor (MPL), and granulocyte colony-stimulating factor receptor have all been implicated.¹⁵⁵ The third hypothesis suggests that the phenotype depends on the level of JAK2 tyrosine kinase activity, called the *dosage effect*. Patients diagnosed with PV showed greater tyrosine kinase activity than patients presenting with an ET phenotype. Experiments in which erythroid progenitors were collected from these patients and tested in colony-forming assays showed that nearly all the cell cultures developing a PV phenotype were homozygous for the JAK2 mutation, whereas the ET phenotype was observed in the vast majority of cell cultures expressing a heterozygous genotype.^{156,157} This phenomenon was corroborated in experiments with transgenic mice.^{158,159} The last hypothesis proposes that a pre-JAK2 mutation produces a premalignant clone,¹⁶⁰⁻¹⁶² predisposing the hematopoietic stem cell to a particular phenotype, and that the JAK2 mutation drives the malignant transformation. Groups have reported mutations coexisting with the JAK2 V617F mutation, like BCR/ABL,^{163,164} MPL mutations,¹⁶⁵ and another version of the JAK2 mutation.^{166,167} Familial MPN provides the strongest support for a pre-JAK2 mutation.^{168,169}

The most appealing model includes all of the hypotheses previously presented and suggests that ET, PV, and PMF may represent a continuum of diseases. It seems reasonable to assume that a pre-JAK2 mutation occurs in the hematopoietic stem cell most if not all of the time to create a hyperproliferative clone that predisposes to additional mutations like the JAK2 mutation. The pre-JAK2 mutation can be familial, congenital, or somatic. Because MPL is expressed in high levels on

megakaryocyte precursors, one *JAK2* V617F mutation (heterozygous) is sufficient to induce MPL signaling and thus stimulate megakaryocyte production. This could lead to the ET phenotype. In contrast, the erythropoietin receptor is expressed in low density on the surface of erythroid precursors, which requires the higher amount of *JAK2* V617F tyrosine kinase that is produced by two *JAK2* mutations (homozygous). This could lead to the PV phenotype. Because MPL stimulation begins with the first *JAK2* mutation and continues with the second *JAK2* mutation, the MPL receptor undergoes continuous stimulation. It has been shown that excessive thrombopoietin stimulation leads to myelofibrosis, which may result in a progression to PMF.¹⁷⁰ The identification of additional mutations in the *BCR/ABL1* negative MPNs to include negative regulators of signaling pathways (*LNK*, *c-CBL*, *SOCs*), tumor suppressor genes (*IZF1*, *TP53*), and epigenetic regulators (*TET2*, *IDH1/2*, *ASXL1*, *EZH2*) combines to set the disease on a particular course. *JAK2* and MPL mutations serve as the drivers for the disease, but mutations in the negative regulators of signaling pathways may synergize with the driver mutations. Mutations in the epigenetic regulator genes may be early events that precede *JAK2* but can also appear late to promote progression. Tumor suppressor gene mutations tend to occur during phases of disease progression.⁶² More than likely, most, if not all, of the hypotheses previously described function together to drive the *BCR/ABL1* negative MPNs down a particular phenotype and through the phases of clonal expansion and disease progression.

OTHER MYELOPROLIFERATIVE NEOPLASMS

Chronic Neutrophilic Leukemia

Chronic neutrophilic leukemia (CNL) is a clonal disorder in which a hyperproliferation of neutrophilic cells in the bone marrow produces sustained neutrophilia in the peripheral blood and hepatosplenomegaly. CNL must be differentiated from CML, based on the absence of the Philadelphia chromosome and the *BCR/ABL* fusion gene, as well as from both a reactive neutrophilic process and other MPNs.¹⁷¹

Incidence

The incidence of CNL is not known, but it is a rare disorder of which about 150 cases have been reported. However, if the WHO criteria had been applied in these cases, many might have been reclassified as reactive rather than neoplastic conditions.¹⁷¹

Clinical Presentation

Hepatosplenomegaly is the most common finding, but 25% to 30% of patients report bleeding from mucocutaneous sites like the gastrointestinal tract. Other symptoms include gout from WBC turnover and pruritus that may be associated with neutrophil infiltration of tissues and organs.¹⁷¹

Peripheral Blood and Bone Marrow

Patients have a WBC count of more than $25 \times 10^9/L$ with a slight left shift. Neutrophils dominate, but the increase in bands, metamyelocytes, myelocytes, and promyelocytes in combination usually comprise fewer than 5% of WBCs but can

be as many as 10%. Neutrophils do not appear dysplastic, but they often contain toxic granules. RBC and platelet morphology are normal in the peripheral blood.¹⁷¹

The bone marrow reflects the peripheral blood in that it is hypercellular with predominantly a proliferation of neutrophils, including myelocytes, metamyelocytes, bands, and segmented neutrophils. The myeloid-to-erythroid ratio is at least 20:1. RBCs and platelets are normal in number, and no cell line exhibits significant dysplastic morphology.¹⁷¹

Diagnosis

The WBC count must be greater than $25 \times 10^9/L$, with greater than 90% mature neutrophils, fewer than 10% immature neutrophilic cells, and fewer than 1% blasts in the peripheral blood. The bone marrow shows an increase in normal-appearing neutrophilic cells with fewer than 5% myeloblasts. Megakaryocytes are normal or slightly left-shifted. Splenomegaly must be present and is often accompanied by hepatomegaly. Reactive neutrophilia must be excluded by eliminating infection, inflammation, and tumors as a cause of the neutrophilia. A diagnosis of CNL can still be made in the presence of a reactive process if clonality of the myeloid line can be documented by karyotyping or molecular analysis. There must be no evidence of the Philadelphia chromosome, *BCR/ABL* mutation, or rearrangements of the *PDGFRA*, *PDGFRB*, or *FGRF1* genes. Lastly, there can be no evidence of PV, PMF, ET, MDS, or MDS/MPN disorders.¹⁷¹

Genetics

Approximately 90% of CNL patients have a normal karyotype, but chromosomal abnormalities are observed, particularly as the disease progresses, including +8, +9, +21, del(20q), del(11q), and del(12p). The Philadelphia chromosome or a *BCR/ABL* mutation cannot be expressed in CNL; otherwise, a diagnosis of CML is required. *JAK2* mutations have been observed, but rarely.¹⁷¹

Prognosis

CNL is a slow, smoldering condition, and patient survival ranges from as short as 6 months to longer than 20 years. The neutrophilia does progress, and some patients develop myelodysplasia and can experience transformation into AML.¹⁷¹

Chronic Eosinophilic Leukemia, Not Otherwise Specified

Chronic eosinophilic leukemia (CEL) is a clonal proliferation of eosinophils from eosinophil precursors that dominate in the bone marrow and peripheral blood. Eosinophils are found in other peripheral tissues, including heart, lungs, central nervous system, gastrointestinal tract, and skin. Hepatosplenomegaly is observed in approximately 30% to 50% of patients. Infiltrating eosinophils degranulate to release cytokines, enzymes, and other granular proteins that damage the surrounding tissue, which results in organ dysfunction.¹⁷²

Clinical Presentation

Although some patients may be asymptomatic when found to have eosinophilia, most have signs and symptoms of fever, fatigue, cough, angioedema, muscle pain, and pruritus. A more

severe sequela of CEL involves the heart. Fibrosis can form in the heart (endomyocardial fibrosis), which can evolve into cardiomegaly. Within the heart, scar tissue may form in the mitral and tricuspid valves, affecting valve function and predisposing to thrombi formation. Other serious complications include peripheral neuropathy, central nervous system dysfunction, pulmonary symptoms from eosinophilic infiltrates, and rheumatologic problems.¹⁷²

Peripheral Blood and Bone Marrow

Peripheral eosinophilia must be observed, with the majority of eosinophils appearing normal. Some evidence of eosinophil abnormality is found, however, and includes the presence of eosinophilic myelocytes and metamyelocytes, hypogranulation, and vacuolization. Neutrophilia is a common finding; other features such as mild monocytosis, basophilia, and the presence of blasts are less common. The bone marrow is hypercellular owing to eosinophilic proliferation and can demonstrate Charcot-Leyden crystals. Myeloblast numbers are elevated but below the 20% threshold necessary to classify the disorder as an acute leukemia. Erythrocytes and megakaryocytes are normal in number but sometimes demonstrate dysplastic morphologic features. Bone marrow fibrosis occurs due to the release of eosinophilic basic protein and eosinophilic cationic proteins from the eosinophil granules. Bone marrow fibrosis contributes to the premature release of eosinophils into the circulation, and they deposit in a variety of tissues.¹⁷²

Diagnosis

The diagnosis of CEL requires eosinophilia with a count of more than 1.5×10^9 cells/L and the presence of malignant features, and the elimination of reactive eosinophilia and other malignancies that have concomitant eosinophilia. Reactive conditions like parasitic infections, allergies, Loeffler syndrome (pulmonary disease), cyclical eosinophilia, angiolymphoid hyperplasia of the skin, collagen vascular disorders, and Kimura disease must be excluded in the differential diagnosis. Likewise, other malignancies that can produce a concomitant eosinophilia include T cell lymphoma, Hodgkin lymphoma, systemic mastocytosis, chronic myelomonocytic leukemia, atypical CML, and ALL. These disorders lead to the release of a variety of interleukins that can drive a secondary eosinophil reaction. No single genetic abnormality is specific for CEL, but CEL can be ruled out by the presence of several karyotypic abnormalities, such as *PDGFRA*, *PDGFRB*, *FGFR1*, and *BCR/ABL*. Common myeloid mutations like +8, i(17q), and *JAK2* can support a diagnosis of CEL.¹⁷²

Prognosis

Survival is variable, but approximately 80% of patients will live 5 years after diagnosis. Features of dysplasia, an increase in karyotype abnormalities, or an increase in blasts indicates an unfavorable prognosis.¹⁷²

Mastocytosis

Mastocytosis is a broad term referring to a clonal neoplastic proliferation of mast cells, which accumulate in one or more

organ systems, but it can present differently and manifest in a range of severities. The WHO group has classified mastocytosis into seven subcategories: cutaneous mastocytosis, indolent systemic mastocytosis, systemic mastocytosis with associated clonal hematologic non–mast-cell-lineage disease, aggressive systemic mastocytosis, mast cell leukemia, mast cell sarcoma, and extracutaneous mastocytoma.¹⁷³

Incidence

Mastocytosis can occur at any age, with cutaneous mastocytosis seen most often in children. Babies can be born with cutaneous mastocytosis, and half of affected children develop the disease before 6 months of age. In contrast, systemic mastocytosis generally occurs after the second decade of life. Approximately 80% of patients with mastocytosis show skin involvement regardless of the type of mastocytosis diagnosed. Cutaneous mastocytosis occurs in the skin; systemic mastocytosis usually involves the bone marrow and other organ systems like the spleen, lymph nodes, liver, and gastrointestinal tract; and mast cell leukemia is characterized by mast cells in the peripheral blood.¹⁷³

Clinical Presentation

Patients present with urticarial lesions (wheel and flare) that may become activated when stroked upon physical examination. Skin lesions also tend to have melanin pigmentation. Four categories of symptom severity have been described in mastocytosis: constitutional systems like fatigue and weight loss; skin manifestations; mediator-related systemic events such as abdominal pain, gastrointestinal distress, headache, and respiratory symptoms; and musculoskeletal complaints like bone pain, arthralgias, and myalgias. Hematologic findings include anemia, leukocytosis, eosinophilia, neutropenia, and thrombocytopenia. In patients with systemic mastocytosis with associated clonal hematologic non–mast-cell-lineage disease the most common associated hematologic finding is chronic myelomonocytic leukemia, but any myeloid or lymphoid malignancy can occur, although myeloid versions predominate.¹⁷³

Diagnosis

The typical skin lesion is the first diagnostic clue to mastocytosis. Cutaneous mastocytosis occurs in three forms: urticaria pigmentosa, diffuse cutaneous mastocytosis, and mastocytosis of the skin, all of which occur predominantly in children. In urticaria pigmentosa, mast cells are confined to the skin and form aggregates in the dermis, whereas in diffuse cutaneous mastocytosis, mast cells are found in more than one cutaneous location. In systemic mastocytosis, mast cells are observed in one area of the bone marrow with fibrosis, other areas of the bone marrow are hypercellular with panmyelosis, and/or mast cells are identified in other extracutaneous sites. In addition to the major criteria just described, at least one of four minor criteria must be met. These include (1) more than 25% of mast cells must be immature or have atypical morphology, like a spindle shape; (2) mast cells must express a *KIT* mutation at codon 816; (3) mast cells must express normal markers and CD2 and/or CD25; and (4) total serum tryptase must be above

20 mg/mL. Key diagnostic features distinguish the six types of systemic mastocytosis. Indolent systemic mastocytosis is characterized by a low–mast cell burden. Systemic mastocytosis with associated clonal hematologic non–mast-cell-lineage disease presents with myelodysplastic syndrome, myeloproliferative neoplasm, AML, lymphoma, or another hematopoietic neoplasm. Aggressive systemic mastocytosis usually does not manifest with skin lesions or mast cells in circulation but does have mast cells in bone marrow, dysplastic hematopoietic changes, and/or hepatosplenomegaly. Mast cell leukemia is characterized by more than 20% atypical mast cells in the bone marrow and more than 10% in the peripheral blood. Mast cell sarcoma presents as a single unifocal mast cell tumor with a high-grade pathology. Extracutaneous mastocytoma also exhibits a unifocal mast cell tumor, but it is of low-grade pathology.¹⁷³

Genetics

The most common genetic mutation in patients with mastocytosis involves codon 816 in the *KIT* gene and occurs in about 95% of adults and 33% of children with systemic mastocytosis. This mutation replaces aspartic acid with valine, which alters the tyrosine kinase receptor activity so as to cause constitutive kinase activity in the absence of ligand. Usually the mutation is somatic, but a few cases of familial *KIT* mutations have been reported. Additional mutations can push proliferation of hematopoietic clones, causing systemic mastocytosis with associated clonal hematologic non–mast-cell-lineage disease. These include mutations of *RUNX1/RUNX1T1* in AML, *JAK1* in MPN, and *FIP1L1/PDGFR*A in myeloid neoplasms with eosinophilia.¹⁷³

Prognosis

Cutaneous mastocytosis in children has a favorable prognosis and may regress spontaneously around puberty. Milder versions like cutaneous mastocytosis and indolent cutaneous mastocytosis follow a benign course and are associated with a

normal life span. Hematologic involvement usually evolves into the corresponding hematologic disease. Patients with aggressive systemic mastocytosis, mast cell leukemia, and mast cell sarcoma are often treated with cytoreductive chemotherapy but may survive only a few months after diagnosis. Signs and symptoms that predict a poorer prognosis include elevated lactate dehydrogenase and alkaline phosphatase, anemia, thrombocytopenia, abnormal peripheral blood morphology, bone marrow hypercellularity, and hepatosplenomegaly.¹⁷³

Myeloproliferative Neoplasm, Unclassifiable

The category *myeloproliferative neoplasm, unclassifiable* (MPN-U) is designed to capture disorders that clearly express myeloproliferative features but either fail to meet the criteria of a specific condition or have features that overlap two or more specific conditions. Most patients with MPN-U fall into one of three groups: patients with an early stage of PV, ET, or PMF in which the criteria that define the disorders are not yet fully developed; patients presenting with features indicative of advanced disease resulting from clonal evolution that masks the potential underlying condition; and patients who have clear evidence of an MPN but who have a concomitant condition like a second neoplasm or an inflammatory condition that alters the MPN features. MPN-U may account for as many as 10% to 15% of MPN disorders, but caution should be exercised so that morphologic changes caused by the patient's cytotoxic drug therapy or growth factor therapy or poor collection of samples are not confused with the features of MPN-U. In patients with MPN-U in the early stages of development or with a concomitant disorder like inflammation, the MPN-U may be reclassified to a specific category of MPN once the disease begins to express typical features or the secondary condition subsides. Likewise, in patients with an advanced MPN, the disorder may be reclassified as an acute leukemia once the blast criterion of more than 20% blasts in the bone marrow is met.¹⁷⁴

SUMMARY

- MPNs are clonal hematopoietic stem cell disorders that result in excessive production and overaccumulation of erythrocytes, granulocytes, and platelets in some combination in bone marrow, peripheral blood, and body tissues.
- Within the classification of MPN, the four major conditions are CML, PV, ET, and PMF.
- In CML, there are large numbers of myeloid precursors in the bone marrow, peripheral blood, and extramedullary tissues.
- The peripheral blood exhibits leukocytosis with increased myeloid series, particularly the later maturation stages, often with increases in eosinophils and basophils.
- The LAP score is dramatically decreased in CML.
- The Philadelphia chromosome, t(9;22), either at the chromosomal or molecular level, must be present in all cases of CML.
- In CML, the bone marrow exhibits intense hypercellularity with a predominance of myeloid precursors. Megakaryocyte numbers are normal to increased.
- Patients with CML progress from a chronic stable phase through an accelerated phase into transformation to acute leukemia.
- Bone marrow transplantation has been successful in CML, and imatinib mesylate, a tyrosine kinase inhibitor, produces remission in most cases.
- Approximately 4% of CML patients given imatinib as first-line therapy develop imatinib resistance.
- Dosage escalation or administration of second-generation tyrosine kinase inhibitors restores remission in most patients with imatinib resistance.
- PV manifests with panmyelosis in the bone marrow with increases in erythrocytes, granulocytes, and platelets.
- The clinical diagnosis of PV requires a hemoglobin of greater than 18.5 g/dL in men and greater than 16.5 g/dL in women or other evidence of increased RBC volume and the presence of *JAK2* V617F or another mutation in the *JAK2* gene. If only one of these major criteria is met, two of the minor criteria must be satisfied.

- The *JAK2*V617F mutation is found in 90% to 95% of PV patients and contributes to the pathogenesis of the disease.
- PV is currently treated with phlebotomy, hydroxyuria, and low-dose aspirin, and then with *JAK2* inhibitors in the future.
- ET involves an increase in megakaryocytes with a sustained platelet count greater than $450 \times 10^9/L$.
- Other diagnostic criteria include normal RBC mass, stainable iron in the bone marrow, absence of the Philadelphia chromosome, lack of marrow collagen fibrosis, absence of splenomegaly or leukoerythroblastic reaction, and absence of any known cause of reactive thrombocytosis.
- In the early phases of ET, peripheral blood shows increased numbers of platelets with abnormalities in size and shape. Bone marrow megakaryocytes are increased in number and in size.
- Complications of ET include thromboembolism and hemorrhage.
- The *JAK2*V617F mutation is observed in 50% to 60% of patients with ET and PMF and contributes to the pathogenesis of the disorders.
- PMF manifests with ineffective hematopoiesis, sparse areas of marrow hypercellularity (especially with increased megakaryocytes), bone marrow fibrosis, splenomegaly, and hepatomegaly.
- The peripheral blood in PMF exhibits immature granulocytes and nucleated RBCs; teardrop-shaped cells are a common finding.
- Platelets may be normal, increased, or decreased in number with abnormal morphology. Micromegakaryocytes may be present.
- Immune responses are altered in about 50% of patients.
- Treatment of PMF includes a variety of approaches to include transfusions, hydroxyuria, INF- γ , busulfan, androgens, erythropoietin, and others.
- *JAK* inhibitors improve splenomegaly and constitutional symptoms in patients with PMF to a greater degree than in ET or PV.
- Other MPNs include CNL, CEL not otherwise specified, mastocytosis, and unclassifiable MPN.

Now that you have completed this chapter, go back and read again the case study at the beginning and respond to the questions presented.

REVIEW QUESTIONS

Answers can be found in the Appendix.

1. A peripheral blood film that shows increased neutrophils, basophils, eosinophils, and platelets is highly suggestive of:
 - a. AML
 - b. CML
 - c. MDS
 - d. Multiple myeloma
2. Which of the following chromosome abnormalities is associated with CML?
 - a. t(15;17)
 - b. t(8;14)
 - c. t(9;22)
 - d. Monosomy 7
3. A patient has a WBC count of $30 \times 10^9/L$ and the following WBC differential:

Segmented neutrophils—38%
Bands—17%
Metamyelocytes—7%
Myelocytes—20%
Promyelocytes—10%
Eosinophils—3%
Basophils—5%

Which of the following test results would be helpful in determining whether the patient has CML?

 - a. Nitroblue tetrazolium reduction product increased
 - b. Myeloperoxidase increased
 - c. Periodic acid-Schiff staining decreased
 - d. FISH positive for *BCR/ABL1* fusion
4. A patient in whom CML has previously been diagnosed has circulating blasts and promyelocytes that total 30% of leukocytes. The disease is considered to be in what phase?
 - a. Chronic stable phase
 - b. Accelerated phase
 - c. Transformation to acute leukemia
 - d. Temporary remission
5. The most common mutation found in patients with primary PV is:
 - a. *BCR/ABL*
 - b. Philadelphia chromosome
 - c. *JAK2* V617F
 - d. t(15;17)
6. The peripheral blood in PV typically manifests:
 - a. Erythrocytosis only
 - b. Erythrocytosis and thrombocytosis
 - c. Erythrocytosis, thrombocytosis, and granulocytosis
 - d. Anemia and thrombocytopenia
7. A patient has a platelet count of $700 \times 10^9/L$ with abnormalities in the size, shape, and granularity of platelets; a WBC count of $12 \times 10^9/L$; and hemoglobin of 11 g/dL. The Philadelphia chromosome is not present. The most likely diagnosis is:
 - a. PV
 - b. ET
 - c. CML
 - d. Leukemoid reaction

8. Complications of ET include all of the following *except*:
 - a. Thrombosis
 - b. Hemorrhage
 - c. Seizures
 - d. Infections
9. Which of the following patterns is characteristic of the peripheral blood in patients with PMF?
 - a. Teardrop-shaped erythrocytes, nucleated RBCs, immature granulocytes
 - b. Abnormal platelets only
 - c. Hypochromic erythrocytes, immature granulocytes, and normal platelets
 - d. Spherocytes, immature granulocytes, and increased numbers of platelets
10. The myelofibrosis associated with PMF is a result of:
 - a. Apoptosis resistance in the fibroblasts of the bone marrow
 - b. Impaired production of normal collagenase by the mutated cells
 - c. Enhanced activity of fibroblasts owing to increased stimulatory cytokines
 - d. Increased numbers of fibroblasts owing to cytokine stimulation of the pluripotential stem cells

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Myelodysplastic Syndromes

34

Bernadette F. Rodak

OUTLINE

Etiology

Morphologic Abnormalities in Peripheral Blood and Bone Marrow

Dyserythropoiesis

Dysmyelopoiesis

Dysmegakaryopoiesis

Differential Diagnosis

Abnormal Cellular Function

Classification of Myelodysplastic Syndromes

French-American-British Classification

World Health Organization Classification

Myelodysplastic/Myeloproliferative Neoplasms

Chronic Myelomonocytic Leukemia

Atypical Chronic Myeloid Leukemia, BCR/ABL1 Negative

Juvenile Myelomonocytic Leukemia

Myelodysplastic/Myeloproliferative Neoplasm, Unclassifiable

Cytogenetics, Molecular Genetics, and Epigenetics

Cytogenetics

Molecular Alterations

Epigenetics

Prognosis

Treatment

Future Directions

OBJECTIVES

After completion of this chapter, the reader will be able to:

1. Define myelodysplastic syndromes (MDSs).
2. Explain the etiology of MDS.
3. Recognize morphologic features of dyspoiesis in bone marrow and peripheral blood.
4. Discuss abnormal functions of granulocytes, erythrocytes, and thrombocytes in MDS.
5. Correlate peripheral blood, bone marrow, and cytogenetic and molecular findings in MDS with classification systems.
6. Compare and contrast the French-American-British and the 2008 World Health Organization classifications of MDS.
7. Discuss prognostic indicators in MDS.
8. Indicate modes of management for MDS.
9. Review the epidemiology of MDS and apply it as a contributor in differential diagnosis.
10. Suggest laboratory tests and their results that would rule out MDS in the differential diagnosis.
11. Explain the rationale for the category of myelodysplastic/myeloproliferative neoplasms (MDS/MPN).
12. Correlate peripheral blood, bone marrow, and cytogenetic findings in MDS/MPN with disease classification.
13. Review prognostic indicators in MDS.
14. Discuss treatment in MDS, including novel therapies.

CASE STUDY

After studying the material in this chapter, the reader should be able to respond to the following case study:

A 43-year-old man experienced fatigue and malaise. He presented with pancytopenia (WBC count of $2.2 \times 10^9/L$, hemoglobin of 6.1 g/dL, platelet count of $51 \times 10^9/L$). The WBC differential was essentially normal. Mean cell volume was 132 fL (reference range, 80 to 100 fL), and vitamin B₁₂ and folate levels were normal. The bone marrow was normocellular with a myeloid-to-erythroid ratio of 1:1 and adequate megakaryocytes. The erythroid component was dysplastic with megaloblastic features. No abnormal localization of immature precursors was noted. Chromosome analysis indicated direct duplication of chromosome 1q. The patient was maintained with transfusions over the next 6 years. At that time, his bone marrow revealed increased erythropoiesis, decreased granulopoiesis, and megakaryopoiesis, all with dysplastic changes. There were 50% to 60% ring sideroblasts.

1. What should be included in the differential diagnosis of patients with pancytopenia and elevated mean cell volume?
2. Given the normal vitamin B₁₂ and folate levels, what is the patient's probable diagnosis?
3. In which WHO 2008 classification does this disorder belong?

For decades, laboratory professionals have observed a group of morphologic abnormalities in peripheral blood films and bone marrow smears of elderly patients. The findings were heterogeneous and affected all cell lines, and the condition either remained stable for years or progressed rapidly to death.

Historically, this pattern of abnormalities was referred to as *refractory anemia*, *smoldering leukemia*, *oligoblastic leukemia*, or *preleukemia*.¹⁻³ In 1982 the French-American-British (FAB) Cooperative Leukemia Study Group proposed terminology and a specific set of morphologic criteria to describe what are now known as *myelodysplastic syndromes* (MDSs).⁴ In 1997 a

group from the World Health Organization (WHO) proposed a new classification that included molecular, cytogenetic, and immunologic criteria in addition to morphologic features.^{5,6} The WHO classification was revised in 2008. Both the FAB and WHO classifications are discussed in this chapter.

MDSs are a group of acquired clonal hematologic disorders characterized by progressive cytopenias in the peripheral blood, reflecting defects in erythroid, myeloid, and/or megakaryocytic maturation.^{7,8} The median age at diagnosis is 70. MDSs rarely affect individuals younger than age 50 unless preceded by chemotherapy or radiation for another malignancy.^{1,9} Cases in young adults and children have been reported, however.^{10,11} The incidence of these disorders seems to be increasing, but this apparent increase may be attributable in part to improved techniques for identifying these diseases and to improved classification.¹²⁻¹⁴ At this time, the fastest-growing segment of the population is the group older than 60 years of age. MDSs are becoming a more common finding in the hematology laboratory, and familiarity with these disorders is an essential part of the body of knowledge of all medical laboratory professionals.

ETIOLOGY

MDS may arise *de novo* (primary MDS) or as a result of therapy (therapy-related MDS). Although MDSs are a group of heterogeneous diseases, all are the result of proliferation of abnormal stem cells.^{7,8,15} The initiating defect in most cases is at the level of the myeloid stem cell, because primarily the erythroid, myeloid, and megakaryocytic cells are affected. It may be that the affected hematopoietic stem cell has lost its lymphopoietic potential, because only rarely does MDS transform to acute lymphoblastic leukemia.^{16,17} The abnormal stem cell may be the result of the cumulative effects of environmental exposure in susceptible individuals. Mutations may be caused by chemical insult, radiation, or viral infection. There also may be an association with smoking.¹⁸ An association with inherited hematologic disorders has also been found.¹⁹ The mutated stem cell produces a pathologic clone of cells that expands in size at the expense of normal cell production.²⁰ Because each mutation produces a unique clone with a specific cellular defect, MDSs have a multitude of expressions. Two morphologic findings are common to all types of MDSs, however; the presence of progressive cytopenias despite cellular bone marrow and dyspoiesis in one or more cell lines.

Disruption of apoptosis may be responsible for the ineffective hematopoiesis in MDS.²¹⁻²⁶ Apoptosis (programmed cell death) regulates cell population by decreasing cell survival. In MDS, apoptosis is increased in early disease, when peripheral blood cytopenias are evident. Later in MDS, when progression toward leukemia is apparent, apoptosis has been shown to be decreased, which allows increased neoplastic cell survival and expansion of the abnormal clone.²⁷⁻³⁰ Other important factors include the levels of antiangiogenic cytokines, tumor necrosis factor, and cellular components of the immune system, as well as the interaction between MDS clonal cells and the hematopoietic inductive microenvironment. Patients with MDS have

increased levels of angiogenic growth factors, including vascular endothelial growth factor.^{31,32}

Therapy-related MDS (t-MDS) occurs in patients who have been treated previously with chemotherapy or radiotherapy or both. Median onset of therapy-related MDS varies with the agents used and is usually 4 to 7 years after therapy was initiated.^{20,33} Patients who have received cytokines, such as G-CSF or GM-CSF, for bone marrow stimulation are also at an increased risk for developing t-MDS.³⁴ Therapy-related MDS often is more aggressive and may evolve quickly into acute myeloblastic leukemia (AML).^{20,33,35} The 2008 WHO classification places therapy-related MDSs into the AML category of therapy-related myeloid neoplasms (Chapter 35).

MORPHOLOGIC ABNORMALITIES IN PERIPHERAL BLOOD AND BONE MARROW

In MDS each of the three major myeloid cell lines has dyspoietic morphologic features. The following sections provide descriptions of common abnormal morphologic findings.^{4,9,19} These descriptions are not all-inclusive because of the large number of possible cellular mutations and combinations of mutations.

Dyserythropoiesis

In the peripheral blood, the most common morphologic finding in dyserythropoiesis is the presence of oval macrocytes (Figure 34-1). When these cells are seen in the presence of normal vitamin B₁₂ and folate values, MDS should be included in the differential diagnosis. Hypochromic microcytes in the presence of adequate iron stores also are seen in MDS. A dimorphic red blood cell (RBC) population (Figure 34-2) is another indication of the clonality of this disease. Poikilocytosis, basophilic stippling, Howell-Jolly bodies, and siderocytes also are indications that the erythrocyte has undergone abnormal development.³⁶

Dyserythropoiesis in the bone marrow is evidenced by RBC precursors with more than one nucleus or abnormal nuclear shapes. The normally round nucleus may have lobes or buds. Nuclear fragments may be present in the cytoplasm

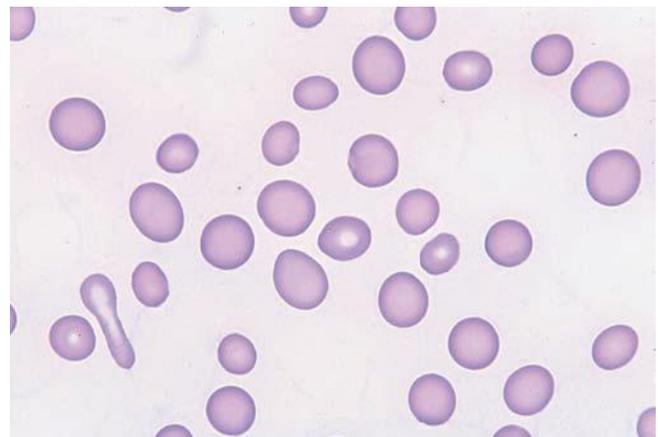


Figure 34-1 Oval macrocytes in peripheral blood ($\times 1000$).

(Figure 34-3). Internuclear bridging is occasionally present (Figure 34-4).³⁷ Abnormal cytoplasmic features may include basophilic stippling or heterogeneous staining (Figure 34-5). Ring sideroblasts are a common finding. Megaloblastoid cellular development in the presence of normal vitamin B₁₂ and folate values is another indication of MDS. The bone

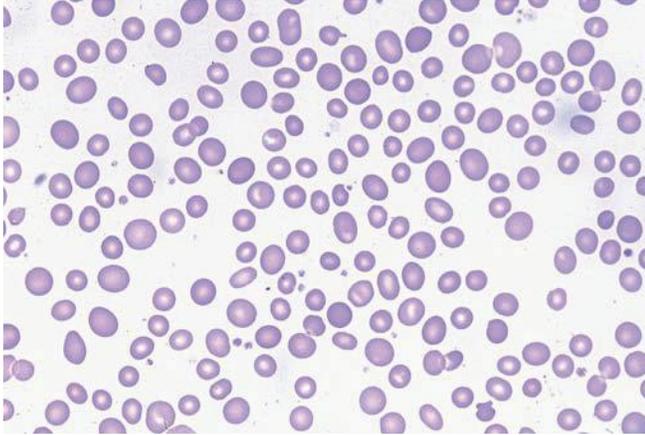


Figure 34-2 Dimorphic erythrocyte population, including macrocytic and microcytic cells (in peripheral blood, $\times 500$).

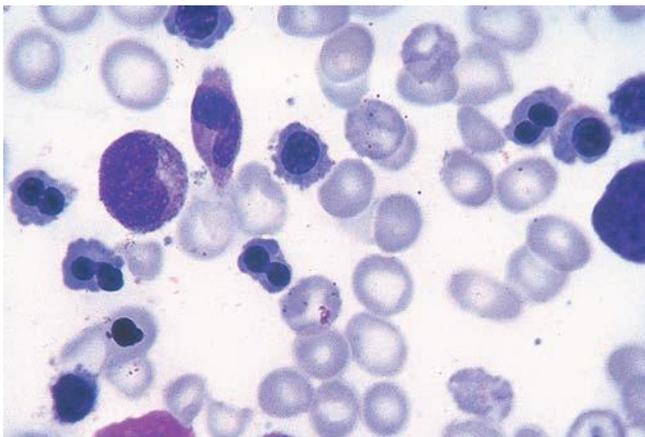


Figure 34-3 Bone marrow specimen showing erythroid hyperplasia and nuclear budding in erythroid precursors ($\times 1000$).

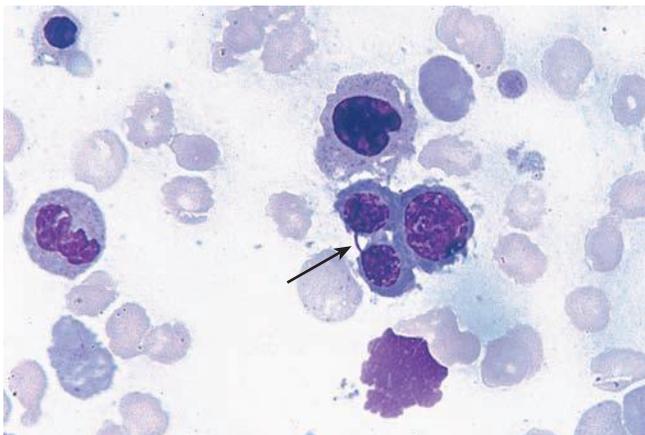


Figure 34-4 Erythroid precursors showing nuclear bridging (arrow) (bone marrow, $\times 1000$).

marrows in these cases may have erythrocytic hyperplasia or hypoplasia (Box 34-1).

Dysmyelopoiesis

Dysmyelopoiesis in the peripheral blood is suspected when there is a persistence of basophilia in the cytoplasm of otherwise mature white blood cells (WBCs), indicating nuclear-cytoplasmic asynchrony (Figure 34-6). Abnormal granulation of the cytoplasm of neutrophils, in the form of larger than normal granules, hypogranulation, or the absence of granules, is a common finding. Agranular bands can be easily misclassified

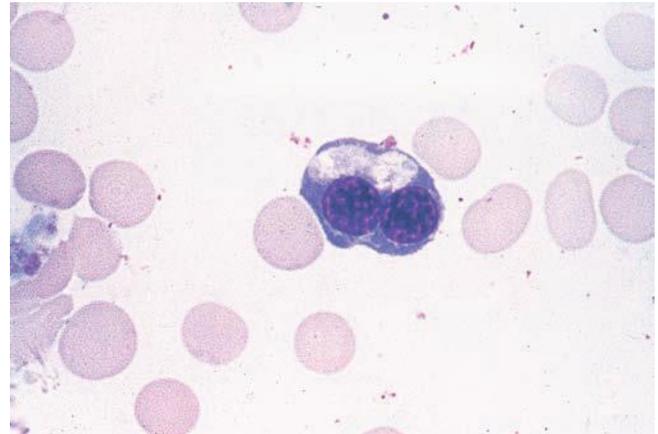


Figure 34-5 Bone marrow specimen showing heterogeneous staining in a bilobed erythroid precursor ($\times 1000$).

BOX 34-1 Morphologic Evidence of Dyserythropoiesis

- Oval macrocytes
- Hypochromic microcytes
- Dimorphic red blood cell (RBC) population
- RBC precursors with more than one nucleus
- RBC precursors with abnormal nuclear shapes
- RBC precursors with uneven cytoplasmic staining
- Ring sideroblasts

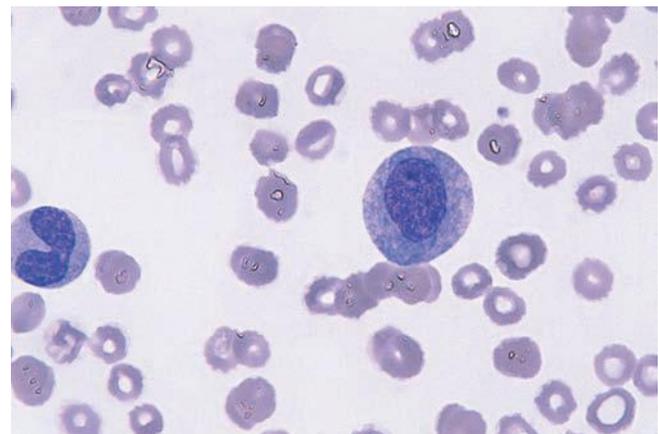


Figure 34-6 This myelocyte (right) in peripheral blood has a nucleus with clumped chromatin and a basophilic immature cytoplasm showing asynchrony. Note also the agranular myeloid cell (left) ($\times 1000$).

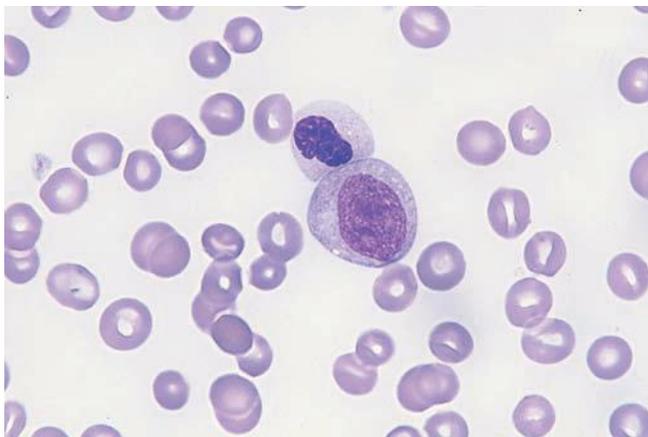


Figure 34-7 Agranular myeloid cells (peripheral blood, $\times 1000$).

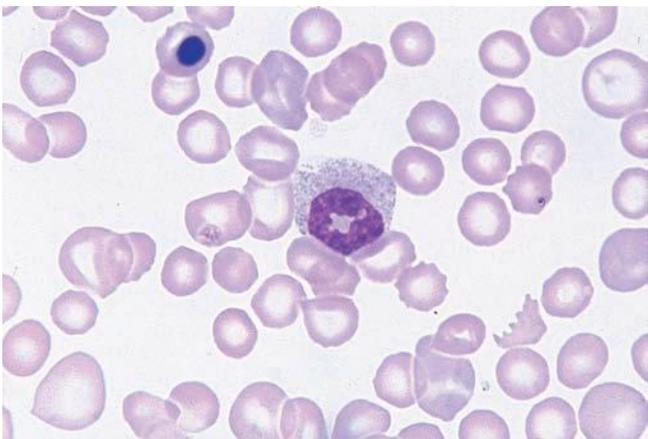


Figure 34-8 Nuclear ring in myeloid cell (peripheral blood, $\times 1000$).

as monocytes (Figure 34-7). Abnormal nuclear features may include hyposegmentation, hypersegmentation, or nuclear rings (Figure 34-8).³⁸

In the bone marrow, dysmyelopoiesis may be represented by nuclear-cytoplasmic asynchrony. Cytoplasmic changes include uneven staining, such as a dense ring of basophilia around the periphery with a clear unstained area around the nucleus or whole sections of cytoplasm unstained, with the remainder of the cytoplasm stained normally (Figure 34-9). There may be abnormal granulation of the cytoplasm in which promyelocytes or myelocytes or both are devoid of primary granules (Figure 34-10), primary granules may be larger than normal, or secondary granules may be reduced in number or absent, and there may be an occasional Auer rod.^{39,40} Agranular promyelocytes may be mistaken for blasts; this could lead to misclassification of the disease in the AML scheme. Abnormal nuclear findings may include hypersegmentation or hyposegmentation and possibly ring-shaped nuclei (Box 34-2).

The bone marrow may exhibit granulocytic hypoplasia or hyperplasia. Monocytic hyperplasia is a common finding in dysplastic marrows.

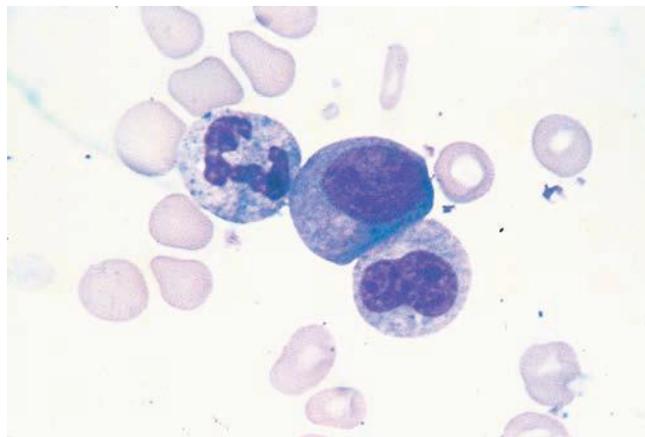


Figure 34-9 Uneven staining of white blood cell cytoplasm (bone marrow, $\times 1000$).

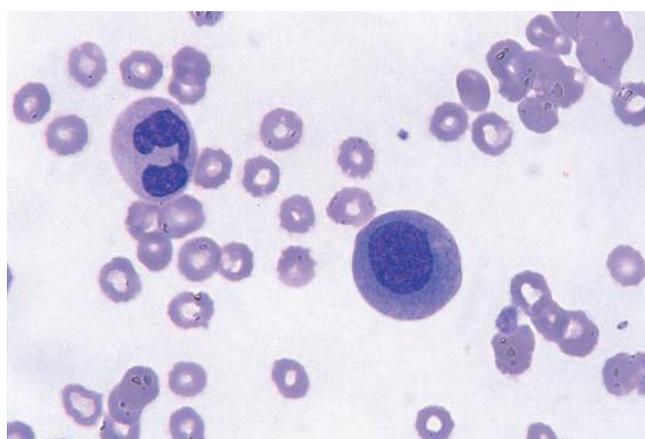


Figure 34-10 Promyelocyte or myelocyte devoid of granules and an agranular neutrophil (bone marrow, $\times 1000$).

BOX 34-2 Morphologic Evidence of Dysmyelopoiesis

- Persistent basophilic cytoplasm
- Abnormal granulation
- Abnormal nuclear shapes
- Uneven cytoplasmic staining

Abnormal localization of immature precursors is a characteristic finding in bone marrow biopsy specimens from patients with MDS.⁴¹ Normally, myeloblasts and promyelocytes reside along the endosteal surface of the bone marrow. In some cases of MDS, these cells tend to cluster centrally in marrow sections.

Dysmegakaryopoiesis

Platelets also exhibit dyspoietic morphology in the peripheral blood. Common changes include giant platelets and abnormal platelet granulation, either hypogranulation or agranulation (Figure 34-11). Some platelets may possess large fused granules. Circulating micromegakaryocytes may be present in peripheral blood from patients with MDS (Figure 34-12).⁹

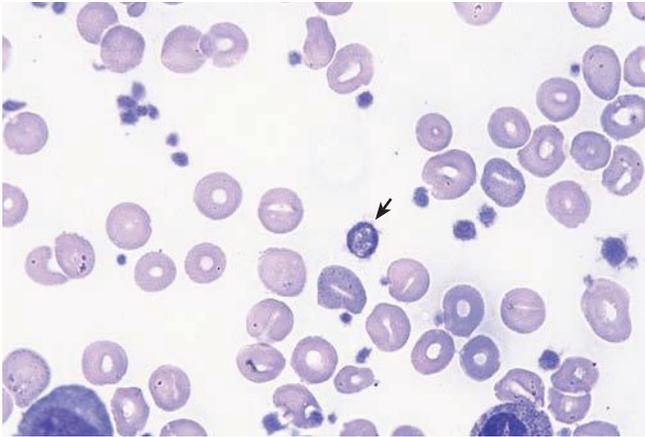


Figure 34-11 Abnormal platelet granulation (arrow) (peripheral blood, $\times 1000$).

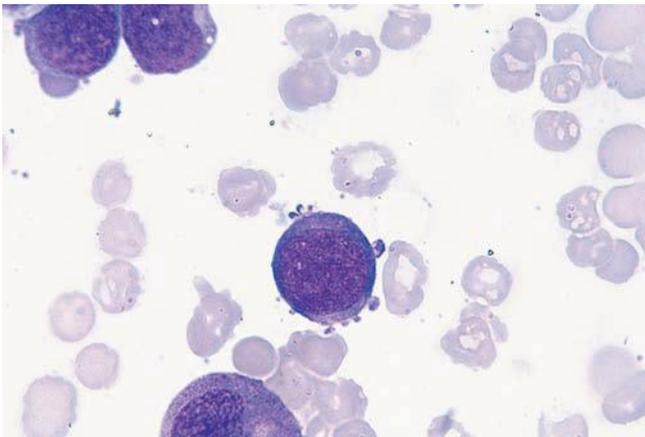


Figure 34-12 Micromegakaryocyte (peripheral blood, $\times 1000$).

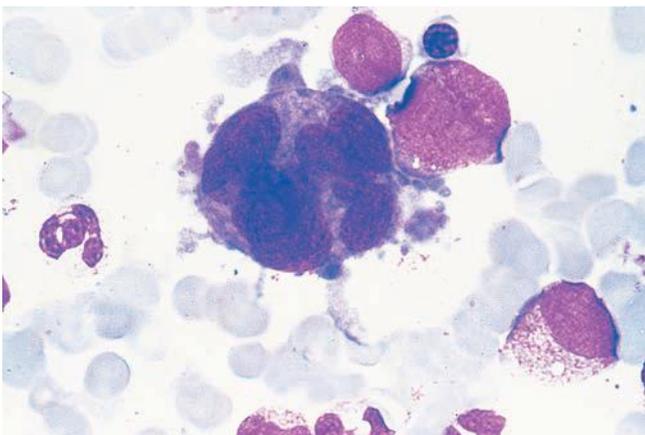


Figure 34-13 Megakaryocyte with small separated nuclei (bone marrow, $\times 1000$).

The megakaryocytic component of the bone marrow may exhibit abnormal morphology: large mononuclear megakaryocytes, micromegakaryocytes, or micromegakaryoblasts. The nuclei in these cells may be bilobed or have multiple small, separated nuclei (Figure 34-13; Box 34-3).⁹

BOX 34-3 Morphologic Evidence of Dysmegakaryopoiesis

- Giant platelets
- Platelets with abnormal granulation
- Circulating micromegakaryocytes
- Large mononuclear megakaryocytes
- Micromegakaryocytes or micromegakaryoblasts or both
- Abnormal nuclear shapes in the megakaryocytes/blasts

DIFFERENTIAL DIAGNOSIS

Dysplasia by itself is not sufficient evidence for MDS, because several other conditions can cause similar morphologic features. Some examples are vitamin B₁₂ or folate deficiency, which can cause pancytopenia and dysplasia, and exposure to heavy metals. Copper deficiency may cause reversible myelodysplasia.⁹ Some congenital hematologic disorders, such as Fanconi anemia and congenital dyserythropoietic anemia, may also present with dysplasia. Parvovirus B19 and some chemotherapeutic agents may give rise to dysplasia similar to that in MDS. Paroxysmal nocturnal hemoglobinuria has similar features, as does human immunodeficiency virus (HIV).⁴² Therefore, a thorough history and physical examination, including questions about exposure to drugs and chemicals, are essential.⁹

ABNORMAL CELLULAR FUNCTION

The cells produced by abnormal maturation not only have an abnormal appearance but also have abnormal function.^{9,43} The granulocytes may have decreased adhesion,^{44,45} deficient phagocytosis,⁴⁵ decreased chemotaxis,^{44,45} or impaired microbicidal capacity.⁴⁶ Decreased levels of myeloperoxidase and alkaline phosphatase may be found.⁴⁷ The RBCs may exhibit shortened survival,⁴⁸ and erythroid precursors may have a decreased response to erythropoietin that may contribute to anemia.⁴⁹ Patients may experience increased bleeding despite adequate platelet numbers.^{9,50,51} The type and degree of dysfunction depend on the mutation present in the hematopoietic stem cell.

CLASSIFICATION OF MYELODYSPLASTIC SYNDROMES

French-American-British Classification

In an effort to standardize the diagnosis of MDSs, the FAB created five classes of MDS, each with a specific set of morphologic criteria. The categories were defined by the amount of dysplasia and the number of blasts in the bone marrow. The diagnosis of acute leukemia required at least 30% blasts in the bone marrow.⁴ The FAB classification included the following:

1. Refractory anemia
2. Refractory anemia with ring sideroblasts (RARS)
3. Refractory anemia with excess blasts (RAEB)
4. Chronic myelomonocytic leukemia (CMML)
5. Refractory anemia with excess blasts in transformation (RAEB-t)⁹

The FAB classification provided a framework for discussion of a seemingly heterogeneous group of disorders; however, its reliance on morphology alone limited its usefulness as a prognostic indicator. In addition, the FAB classification did not view MDSs in their totality because it did not address therapy-related or hereditary forms, and childhood MDS was not considered. Advances in medical knowledge, including molecular analysis, have allowed integration of clinical, immunologic, genetic, and molecular data with morphologic features. The WHO classification retains many of the FAB features, while recognizing molecular, cytogenetic, and immunologic characteristics of these disorders. The WHO classification also removed the problematic categories of CMML and RAEB-t and placed them in MDS/MPD and acute leukemia, respectively.^{19,52}

World Health Organization Classification

The original modifications from the FAB classification of MDS included a reduction in the percentage of blasts required for diagnosis of AML from 30% to 20% and the recognition of two new classifications: refractory cytopenia with multilineage dysplasia

(RCMD) and del(5q) syndrome. The 2008 revision of the WHO criteria added the category of refractory cytopenia with unilineage dysplasia (RCUD), refined some categories, and added the provisional category of childhood MDS, also called *refractory cytopenia of childhood*. The 2008 WHO classification is outlined in [Box 34-4](#) and detailed in [Table 34-1](#). The classification is extensive, and only the highlights are presented in this chapter.¹⁹

BOX 34-4 World Health Organization Classification of Myelodysplastic Syndromes (2008)

Refractory cytopenia with unilineage dysplasia
 Refractory anemia with ring sideroblasts
 Refractory cytopenia with multilineage dysplasia
 Refractory anemia with excess blasts
 Myelodysplastic syndrome with isolated del(5q)
 Myelodysplastic syndrome, unclassifiable
 Childhood myelodysplastic syndrome (provisional)

From Swerdlow SH, Campo E, Harris NL, et al, editors: *WHO classification of tumours of haematopoietic and lymphoid tissues*, ed 4, Lyon, France, 2008, IARC Press.

TABLE 34-1 Peripheral Blood and Bone Marrow Findings in Myelodysplastic Syndromes (MDSs)

Disease	Blood Findings	Bone Marrow Findings
Refractory cytopenia with unilineage dysplasia (RCUD); refractory anemia (RA); refractory neutropenia (RN); refractory thrombocytopenia (RT)	Unicytopenia* No or rare blasts (<1%) [†]	Unilineage dysplasia: ≥10% of cells in one myeloid lineage <5% blasts <15% of erythroid precursors are ring sideroblasts
Refractory anemia with ring sideroblasts (RARS)	Anemia No blasts	≥15% of erythroid precursors are ring sideroblasts Erythroid dysplasia only <5% blasts
Refractory cytopenia with multilineage dysplasia (RCMD)	Cytopenia(s) No or rare blasts (<1%) [†] No Auer rods <1 × 10 ⁹ /L monocytes	Dysplasia in ≥10% of cells in two or more myeloid lineages (neutrophil and/or erythroid precursors and/or megakaryocytes) <5% blasts in marrow No Auer rods ±15% ring sideroblasts
Refractory anemia with excess blasts 1 (RAEB-1)	Cytopenia(s) <5% blasts No Auer rods <1 × 10 ⁹ /L monocytes	Unilineage or multilineage dysplasia 5%–9% blasts [†] No Auer rods
Refractory anemia with excess blasts 2 (RAEB-2)	Cytopenia(s) 5%–19% blasts ± Auer rods [‡] <1 × 10 ⁹ /L monocytes	Unilineage or multilineage dysplasia 10%–19% blasts [†] ± Auer rods [‡]
Myelodysplastic syndrome, unclassified (MDS-U)	Cytopenia(s) ≤1% blasts [†]	Unequivocal dysplasia in <10% of cells in one or more myeloid cell lines when accompanied by a cytogenetic abnormality considered as presumptive evidence for a diagnosis of MDS <5% blasts
MDS associated with isolated del(5q)	Anemia Usually normal or increased platelet count No or rare blasts (<1%)	Normal to increased megakaryocytes with hypolobulated nuclei <5% blasts Isolated del(5q) cytogenetic abnormality No Auer rods

From Swerdlow SH, Campo E, Harris NL, et al, editors: *WHO classification of tumours of haematopoietic and lymphoid tissues*, ed 4, Lyon, France, 2008, IARC Press.

*Bicytopenia may occasionally be observed. Cases with pancytopenia should be classified as MDS-U.

[†]If the marrow myeloblast percentage is less than 5%, but there are 2% to 4% myeloblasts in the blood, the diagnostic classification is RAEB-1. Cases of RCUD and RCMD with 1% myeloblasts in the blood should be classified as MDS-U.

[‡]Cases with Auer rods and less than 5% myeloblasts in the blood and less than 10% myeloblasts in the marrow should be classified as RAEB-2.

Refractory Cytopenia with Unilineage Dysplasia

Presenting symptoms of RCUD are related to the cytopenia—namely, fatigue or shortness of breath if anemia is present; increased infections from neutropenia; and petechiae, bruising, or bleeding if thrombocytopenia is present. This category includes MDS cases with less than 1% blasts in the peripheral blood and less than 5% blasts in the bone marrow. Dysplasia must be present in more than 10% of a single myeloid lineage. Included in RCUD is refractory anemia with only dyserythropoiesis (but less than 15% ring sideroblasts), refractory neutropenia, and refractory thrombocytopenia.⁵² Although cytogenetic abnormalities may be seen in up to 50% of cases of refractory anemia, none is specific to the diagnosis. Median survival is generally 2 to 5 years, with only a 2% risk of transformation to acute leukemia.^{52,53}

Refractory Anemia with Ring Sideroblasts

In RARS, anemia and dyserythropoiesis are present, and more than 15% of the bone marrow erythroid precursors are ring sideroblasts. To be considered a ring sideroblast, an erythroid precursor must contain at least five iron granules per cell, and these iron-containing mitochondria must circle at least one third of the nucleus (Figure 34-14).⁵⁴ In the peripheral blood there may be a dimorphic picture, with a population of hypochromic cells along with a majority of normochromic cells. RARS occurs primarily in the older population. Mean survival is 69 to 108 months.⁵⁵ Refractory anemia with ring sideroblasts and marked thrombocytosis and *JAK2* V617F mutation are discussed with the myelodysplastic/myeloproliferative neoplasms.⁵⁶ Acquired sideroblastic anemia, which is not considered MDS, is discussed in Chapter 20.

Refractory Cytopenia with Multilineage Dysplasia

RCMD is categorized by one or more cytopenias, dysplasia in two or more myeloid cell lines, less than 1% blasts in peripheral blood, and less than 5% blasts in the bone marrow. In RCMD, the myeloblasts do not contain Auer rods; if Auer rods are noted, the disorder is classified as RAEB-2.⁵⁷ Some cases of RCMD have more than 15% ring sideroblasts, but the dyspoiesis in more than the erythroid line places them in the RCMD

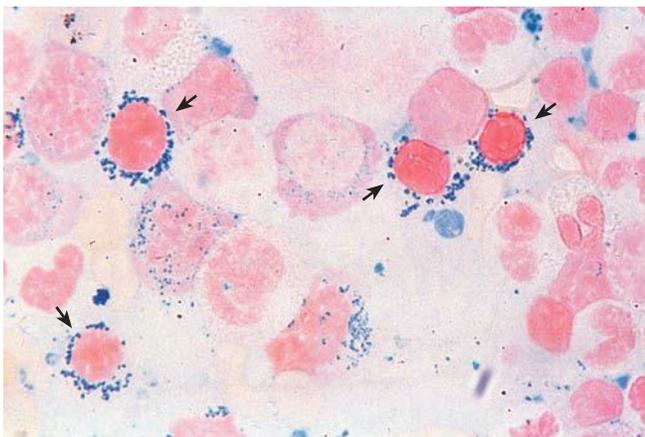


Figure 34-14 Ring sideroblast (arrows) (bone marrow, Prussian blue stain, $\times 1000$).

rather than the RARS category.⁵³ This distinction is important, because RCMD has a more aggressive course than RARS.³³

Refractory Anemia with Excess Blasts

Trilineage cytopenias, as well as significant dysmyelopoiesis, dysmegakaryopoiesis, or both, are common in RAEB. According to the WHO classification, the peripheral blood must contain 2% to 19% blasts. In the bone marrow, blasts number 5% to 19%. RAEB is distinguished from RCMD by myeloblast percentage. Because there are significant differences in survival and because evolution to AML may occur, the WHO classification divided RAEB into two types, depending on the percentage of blasts in blood and bone marrow:

RAEB-1—5% to 9% blasts in the bone marrow or 2% to 4% blasts in the peripheral blood

RAEB-2—10% to 19% blasts in the bone marrow and 5% to 19% blasts in the peripheral blood

The presence of Auer rods, regardless of blast count, qualifies a case as RAEB-2. RAEB with greater than 10% myeloblasts has a more aggressive course, with a greater percentage of cases transforming to AML.^{53,57}

Myelodysplastic Syndrome with Isolated *del(5q)* (*5q– Syndrome*)

In patients who have only the deletion of 5q (*5q–*), MDS represents a fairly well-defined syndrome, affecting predominantly women and occurring at a median age of 67. These patients typically have refractory anemia without other cytopenias and/or thrombocytosis, hypolobulated megakaryocytes, and erythroid hypoplasia.⁵⁸⁻⁶⁰ There are less than 1% blasts in the peripheral blood, and Auer rods are not seen.⁶¹ Patients with MDS with isolated *del(5q)* have long-term stable disease (median survival, 145 months). The thalidomide analogue lenalidomide (Revlimid) has proven to be effective in patients with isolated *del(5q)*, as well as in those with *del(5q)* and additional cytogenetic abnormalities.⁵⁸⁻⁶⁰

Myelodysplastic Syndrome, Unclassifiable

The category of *myelodysplastic syndrome, unclassifiable* refers to subtypes of MDS that initially lack the specific changes necessary for classification into other MDS categories. If characteristics of a specific subtype develop later, the case should be reclassified into the appropriate group.⁶²

Childhood Myelodysplastic Syndromes

De novo MDS in children is very rare, and although some of the characteristics of adult MDS are present, there are also some distinct differences. The 2008 WHO classification introduced a provisional category of *refractory cytopenia of childhood*. Several authors have addressed this provisional category in detail.^{10,52}

MYELODYSPLASTIC/MYELOPROLIFERATIVE NEOPLASMS

The MDS/MPN category includes myeloid neoplasms with clinical, laboratory, and morphologic features that are characteristic

BOX 34-5 Classification of Myelodysplastic Syndromes/Myeloproliferative Neoplasms

Chronic myelomonocytic leukemia
 Atypical chronic myeloid leukemia, *BCR/ABL1* negative
 Juvenile myelomonocytic leukemia
 Myelodysplastic/myeloproliferative neoplasm, unclassifiable

From Swerdlow SH, Campo E, Harris NL, et al, editors: *WHO classification of tumours of haematopoietic and lymphoid tissues*, ed 4, Lyon, France, 2008, IARC Press.

of both MDS and MPN. Included in this classification are chronic myelomonocytic leukemia; atypical chronic myeloid leukemia; juvenile myelomonocytic leukemia; and MDS/MPN, unclassifiable, with a provisional subtype of refractory anemia with ring sideroblasts and thrombocytosis (Box 34-5).⁵²

Chronic Myelomonocytic Leukemia

CMML is characterized by a persistent monocytosis of more than $1.0 \text{ monocyte} \times 10^9/\text{L}$, absence of the *BCR/ABL1* fusion gene, less than 20% blasts and promonocytes in the peripheral blood and bone marrow, and dysplasia in one or more myeloid cell line. Patients usually have an increased leukocyte count with absolute monocytosis. Dysgranulopoiesis is evident, but neutrophil precursors make up less than 10% of the total leukocytes.⁶³ Splenomegaly may be present due to infiltration of leukemic cells. Although cytogenetic abnormalities are found in up to 40% of patients, there is none specific for CMML. Prognosis varies, depending on the number of blasts plus promonocytes. If there are less than 5% blasts and promonocytes in the peripheral blood and less than 10% in the bone marrow, the disease is classified as CMML-1 and the prognosis is better than in those cases in which there are 5% to 19% blasts and promonocytes in the peripheral blood or 10% to 19% in the bone marrow (classified as CMML-2).^{52,63}

Atypical Chronic Myeloid Leukemia, *BCR/ABL1* Negative

Atypical CML, *BCR/ABL1* negative (aCML), is characterized by leukocytosis with morphologically dysplastic neutrophils and their precursors. Basophilia may be present, but it is not a prominent feature. Multilineage dysplasia is common. The *BCR/ABL1* fusion gene is not present, but a variety of other karyotypic abnormalities may be seen. Dyspoiesis may be seen in all cell lines, but it is most remarkable in the neutrophils, which may exhibit Pelger-Huët-like cells, hypogranularity, and bizarre segmentation.^{64,65} The prognosis is poor for patients with aCML, who either progress to AML or succumb to bone marrow failure.⁶⁶

Juvenile Myelomonocytic Leukemia

Juvenile myelomonocytic leukemia is a clonal disorder characterized by proliferation of the granulocytic and monocytic cell lines and affects children from 1 month to 14 years of age. There is a strong association with neurofibromatosis type 1.⁶⁷

Allogeneic stem cell transplantation is effective in about 50% of patients.⁶⁸

Myelodysplastic/Myeloproliferative Neoplasm, Unclassifiable

The designation *MDS/MPN, unclassifiable* is used for cases that meet the criteria for MDS/MPN but do not fit into one of the specified subcategories.⁶⁹ Within this group there is a provisional entity that has features of refractory anemia with ring sideroblasts and thrombocytosis (RARS-T) and also carries the *JAK2* V617F mutation.⁵⁶

CYTOGENETICS, MOLECULAR GENETICS, AND EPIGENETICS**Cytogenetics**

Chromosome abnormalities are found in about 50% of cases of de novo MDS and 90% to 95% of t-MDS.⁷⁰ Karyotype has a major effect on prognosis in MDS patients, and specific karyotypes can be used cautiously to predict response to certain treatments.⁷⁰ Balanced translocations, which are common among patients with AML, are found only rarely in cases of de novo MDS.^{19,70} Except for del(5q), no cytogenetic abnormality is specific to subtype. The most common abnormalities involve chromosomes 5, 7, 8, 11, 13, and 20.^{9,19} The most common single abnormalities are trisomy 8 and monosomy 7.^{71,72} Less common abnormalities in MDS are 12p-, iso 17, -22, and loss of the Y chromosome.⁷³

Molecular Alterations

Advances in molecular genetic testing have made testing more available for routine use, and such information could be used to strengthen other prognostic indicator schemes.⁷⁴ Likewise, identification of genetic defects may allow development of targeted therapies.⁷⁵ Although not specific to MDS, the most common mutations include those in the *TP53* gene,⁷⁴ *RUNX1*,^{76,77} and *TET2*.⁷⁸ *NRAS* has been detected in a small percentage of MDS patients.^{79,80} It appears that a multistep process is required for transformation of MDS to AML. Some gene mutations, such as *TET2*, confer a more favorable prognosis,⁷⁸ while others such as *TP53* confer a higher risk of transformation.⁷⁹

Epigenetics

The term *epigenetics* describes changes in gene expression that occur without altering the DNA sequence. Gene function is affected through selective activation or inactivation, rather than a change in the primary nucleotide sequence itself.^{81,82} In oncogenesis, regions of a gene with specific regulatory functions, such as apoptosis, may be hypermethylated.⁸³ Incorporation of demethylating agents into the DNA appears to slow the progression of MDS, although the mechanism is not clearly understood.⁸²⁻⁸⁴

PROGNOSIS

In 1997 the International Prognostic Scoring System (IPSS) was developed to predict prognosis of patients with primary

TABLE 34-2 Revised International Prognostic Scoring System for MDS

	SCORE VALUE						
	0	0.5	1	1.5	2	3	4
Cytogenetics	Very Good	—	Good	—	Intermediate	Poor	Very Poor
BM Blasts (%)	≤2%	—	>2% to <5%	—	5% to 10%	>10%	—
Hemoglobin (g/dL)	≥10	—	8 to <10	<8	—	—	—
Platelets (×10 ⁹ /L)	≥100	50 to <100	<50	—	—	—	—
ANC (×10 ⁹ /L)	≥0.8	<0.8	—	—	—	—	—
Risk Score and Median Survival (All Ages)							
		Karotype					
Very Low	≤1.5 (8.8 years)	Very Good	-Y, del(11q)				
Low	>1.5 to 3 (5.3 years)	Good	Normal, del(5q), del(12p), del(20q), double incl del 5(q)				
Intermediate	>3 to 4.5 (3.0 years)	Intermediate	Del(7q), +8, +19, i(17q), any other single or double abnormality				
High	>4.5 to 6 (1.6 years)	Poor	-7, inv(3)/t(3q), double incl -7/del(7q), complex 3 abnormalities				
Very High	>6 (0.8 years)	Very Poor	Complex >3 abnormalities				
Median survival is also adjusted for age and decreases with age							

Adapted from Greenberg et al. Revised international prognostic scoring system for myelodysplastic syndromes. *Blood* 2012;120(12):2454-2465.

untreated MDS.⁸⁵ In 2012 a refinement of the IPSS scoring system integrated newer cytogenetic groupings and depth of cytopenias into the equation, defining five major prognostic categories. The basis of the revised system retained three of the original parameters—cytogenetics, bone marrow blast percentage, and cytopenias—but divided the cytogenetic groups into five rather than the original three, split the blast percentage into more groups, and addressed the depth of cytopenias. Other features that affected survival but not transformation into AML included patient age, serum ferritin, patient performance status, and lactate dehydrogenase levels.⁸⁶ Recently, levels of specific cytokines have been shown to affect disease progression in MDS.⁸⁷ Table 34-2 summarizes the revised IPSS.

TREATMENT

Treatment of MDS patients is challenging because many are older and have coexisting illnesses, and the heterogeneity of the disease makes the use of one standard treatment impossible. The overall goal of treatment is to provide improved quality of life and to prolong survival.

Supportive care has been the predominant mode of treatment for most MDS patients, except those who qualify for stem cell transplantation. Supportive care includes administration of blood products (RBCs and platelets as necessary) and prevention or treatment of infections with antibiotics.

Recently, however, three drugs (lenalidomide, azacitidine, and decitabine) have been approved by the U.S. Food and Drug Administration (FDA) that show promise when used either alone or in combination with other therapies.^{84,88} Azacitidine and decitabine belong to a group of drugs that deplete intracellular methyltransferases (DNMTs) and are effective in low dose, with minimal side effects, and have improved the quality of life for patients with high-grade MDS.⁸⁴ Therapies are usually

stratified into those used in low-risk disease and those in higher-risk MDS cases.

Treatment for patients with low-risk MDS is aimed at maintaining residual function of the bone marrow through the use of hematopoietic growth factors such as erythropoietin, thrombopoietin, and granulocyte colony-stimulating factor. Although levels of these growth factors are often normal in MDS patients, there is a subset of patients who respond to their use.⁸⁸⁻⁹⁰

In patients with low-risk MDS, immunosuppressive therapy with drugs such as antithymocyte globulin and cyclosporine has resulted in decreased risk of leukemic transformation.⁹⁰⁻⁹²

Lenalidomide (Revlimid; Celgene, Summit, NJ), a thalidomide analogue that is less toxic than thalidomide, was approved by the FDA in 2005 for use in patients with low- or intermediate-risk MDS.⁹³⁻⁹⁵ It has shown remarkable promise, especially in patients with the 5q chromosome arm deletion. Transfusion independence was achieved in 64% of patients, and a median increase of 3.9 g/dL of hemoglobin was achieved in patients taking the drug. Complete cytogenetic remission was seen in 55% of MDS patients taking lenalidomide, whereas in MDS patients taking erythropoietin, cytogenetic remission is rare.⁹³ Lenalidomide has immunomodulatory and antiangiogenic effects.⁸⁸⁻⁹⁰ The apparent efficacy of lenalidomide must be weighed against its ability to cause significant myelosuppression.^{88,93}

NRAS is mutated in about 20% of MDS patients. Farnesyltransferase inhibitors interfere with this process.^{90,96} Patients with high-risk MDS benefit from treatment with hypomethylating agents such as azacitidine and, to a lesser extent, decitabine.^{81,82,84,88,97-99}

The only cure is hematopoietic stem cell transplantation. Patients with an IPSS score of intermediate 2 or higher and patients with more than 10% blasts should be considered for allogeneic stem cell transplantation.^{100,101} Stem cell transplantation is most successful in patients younger than age 70 with no comorbidity.⁹

Future Directions

As research addressing the role of apoptosis in MDS continues, future therapies may be aimed at controlling apoptosis, with or without the use of chemotherapeutic agents. Because effective treatment for MDS remains limited, it has been suggested that patients be provided with information on the prognosis for

their type of MDS, available therapies, and success rates and should take part in making decisions regarding their treatment.^{102,103} As more is learned about the molecular biology of MDS, it may be possible to develop customized treatment plans for individual patients.¹⁰⁴

SUMMARY

- MDSs are a group of clonal disorders characterized by progressive cytopenias and dyspoiesis of the myeloid, erythroid, and megakaryocytic cell lines.
- The dyspoiesis is evidenced by abnormal morphologic appearance and abnormal function of the cell lines affected.
- The WHO classification of MDSs is based on morphologic, molecular, cytogenetic, and immunologic characteristics of blood cell lines.
- Prognosis in MDS depends on several factors, including percentage of bone marrow blasts, depth of cytopenias, and karyotypic abnormalities.
- Treatment of MDS depends on the prognosis. If the prognosis is favorable, patients may receive only supportive therapy.

- Other treatments that have met with limited success include chemotherapeutic agents and epigenetic modifiers.
- Currently, the only cure for MDS is bone marrow or hematopoietic stem cell transplantation.
- Future treatment possibilities include the use of apoptosis-controlling drugs.

Now that you have completed this chapter, go back and read again the case study at the beginning and respond to the questions presented.

REVIEW QUESTIONS

Answers can be found in the Appendix.

1. MDSs are most common in which age group?
 - a. 2 to 10 years
 - b. 15 to 20 years
 - c. 25 to 40 years
 - d. Older than 50 years
2. What is a major indication of MDS in the peripheral blood and bone marrow?
 - a. Dyspoiesis
 - b. Leukocytosis with left shift
 - c. Normal bone marrow with abnormal peripheral blood features
 - d. Thrombocytosis
3. An alert hematologist should recognize all of the following peripheral blood abnormalities as diagnostic clues in MDS *except*:
 - a. Oval macrocytes
 - b. Target cells
 - c. Agranular neutrophils
 - d. Circulating micromegakaryocytes
4. For an erythroid precursor to be considered a ring sideroblast, the iron-laden mitochondria must encircle how much of the nucleus?
 - a. One quarter
 - b. One third
 - c. Two thirds
 - d. Entire nucleus
5. According to the WHO classification of MDS, what percentage of blasts would constitute transformation to an acute leukemia?
 - a. 5%
 - b. 10%
 - c. 20%
 - d. 30%
6. A patient has anemia, oval macrocytes, and hypersegmented neutrophils. Which of the following tests would be most efficient in differential diagnosis of this disorder?
 - a. Serum iron and ferritin levels
 - b. Erythropoietin level
 - c. Vitamin B₁₂ and folate levels
 - d. Chromosome analysis
7. A 60-year-old woman comes to the physician with fatigue and malaise. Her hemoglobin is 8 g/dL, hematocrit is 25%, RBC count is $2.00 \times 10^{12}/L$, platelet count is $550 \times 10^9/L$, and WBC count is $3.8 \times 10^9/L$. Her WBC differential is unremarkable. Bone marrow shows erythroid hypoplasia and hypolobulated megakaryocytes; granulopoiesis appears normal. Ring sideroblasts are rare. Chromosome analysis reveals the deletion of 5q only. Based on the classification of this disorder, what therapy would be most appropriate?
 - a. Supportive therapy; lenalidomide if the disease progresses
 - b. Aggressive chemotherapy
 - c. Bone marrow transplantation
 - d. Low-dose cytosine arabinoside, accompanied by *cis*-retinoic acid

8. Which of the following is *least* likely to contribute to the death of patients with MDS?
 - a. Neutropenia
 - b. Thrombocytopenia
 - c. Organ failure
 - d. Neuropathy
9. Into what other hematologic disease does MDS often convert?
 - a. Megaloblastic anemia
 - b. Aplastic anemia
 - c. AML
 - d. Myeloproliferative disease
10. Chronic myelomonocytic leukemia is classified in the WHO system as:
 - a. A myeloproliferative neoplasm
 - b. Myelodysplastic syndrome, unclassified
 - c. MDS/MPN
 - d. Acute leukemia

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35

Acute Leukemias

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OUTLINE

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Sudan Black B

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OBJECTIVES

After completion of this chapter, the reader will be able to:

1. Discuss the causes and development of acute leukemia.
2. Characterize the diagnostic criteria used for acute myeloid and acute lymphoblastic leukemias.
3. Compare and contrast acute lymphoblastic and myeloid leukemias by morphology, presenting signs and symptoms, laboratory findings, and prognosis.
4. Interpret the results of diagnostic tests for acute leukemias.
5. Discuss tumor lysis syndrome, including risk, cause, and laboratory findings.
6. Discuss the cell staining patterns for the following tests: myeloperoxidase, Sudan black B, and esterases.

CASE STUDY

After studying the material in this chapter, the reader should be able to respond to the following case study:

A 5-year-old child was seen by her family physician because of weakness and headaches. She had been in good health except for the usual communicable diseases of childhood. Physical examination revealed a pale, listless child with multiple bruises. The WBC count was $15 \times 10^9/L$, the hemoglobin was 8 g/dL, and the platelet count was $90 \times 10^9/L$. She had “abnormal cells” in her peripheral blood (Figure 35-1). Cytogenetic studies revealed hyperdiploidy.

1. What is the most likely diagnosis?
2. What characteristics of this disease indicate a positive prognosis?
3. What prognosis is associated with the hyperdiploidy?

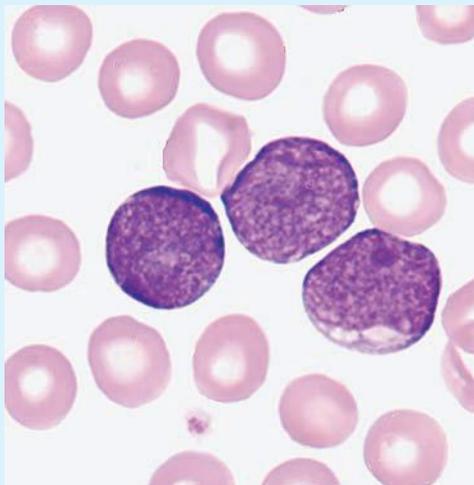


Figure 35-1 Peripheral blood film for the patient in the case study ($\times 1000$). (From Carr JH, Rodak BF: *Clinical hematology atlas*, ed 4, St. Louis, 2013, Saunders.)

INTRODUCTION

The broad term *leukemia* is derived from the ancient Greek words *leukos* (λευκός), meaning “white,” and *haima* (αἷμα), meaning “blood.”¹ As defined today, acute leukemia refers to the rapid, clonal proliferation in the bone marrow of lymphoid or myeloid progenitor cells known as lymphoblasts and myeloblasts, respectively. When proliferation of blasts overwhelms the bone marrow, blasts are seen in the peripheral blood and the patient’s symptoms reflect suppression of normal hematopoiesis.

For most cases of acute leukemia, the causes directly related to the development of the malignancy are unknown. The exceptions that exist are certain toxins that can induce genetic changes leading to a malignant phenotype. Environmental exposures known to lead to hematopoietic malignancies include radiation and exposure to organic solvents, such as benzene. Rarely, leukemias can be seen in patients with known familial cancer predisposition syndromes. Alkylating agents and other forms of chemotherapy used to treat various forms of cancer can induce deoxyribonucleic acid (DNA) damage in hematopoietic cells, leading to therapy-related leukemias.

Regardless of the mechanism of initial genetic damage, the development of leukemia is currently believed to be a stepwise progression of mutations or “multiple hits” involving mutations in genes that give cells a proliferative advantage, as well as mutations that hinder differentiation.^{2,3} These mutations result in transformation of normal hematopoietic stem cells or precursors into leukemic stem cells (LSCs). The LSCs then initiate, proliferate, and sustain the leukemia.⁴

CLASSIFICATION SCHEMES FOR ACUTE LEUKEMIAS

The French-American-British (FAB) classification of the acute leukemias was devised in the 1970s and was based on

morphologic examination along with cytochemical stains to distinguish lymphoblasts from myeloblasts (Figure 35-2). The use of cytochemical stains continues to be a useful adjunct for differentiation of hematopoietic diseases, especially acute leukemias. The details of the cytochemical stains are addressed at the end of this chapter. In addition to morphologic and cytochemical stains, techniques commonly used to diagnose hematopoietic malignancies include flow cytometry and genetic/molecular studies. Findings of these techniques are discussed throughout the chapter in relation to specific leukemias.

Hematologists and pathologists are now moving toward more precise classification of many of the leukocyte neoplasms based on recurring chromosomal and genetic lesions found in many patients. These lesions are related to disruptions of oncogenes, tumor suppressor genes, and other regulatory elements that control proliferation, maturation, apoptosis, and other vital cell functions. In 2001 the World Health Organization (WHO) published new classification schemes for nearly all of the tumors of hematopoietic and lymphoid tissues,⁵ and in some cases WHO melded the older morphologic schemes with the newer schemes. For instance, in the WHO classification scheme for acute myeloid leukemias (AMLs), there are some remnants of the old FAB classification, but new classifications were introduced for leukemias associated with consistently recurring chromosomal translocations. According to the WHO classification, a finding of at least 20% blasts in the bone marrow is required for diagnosis of the majority of acute leukemias, and testing must be performed to detect the presence or absence of genetic anomalies. In 2008 the WHO classification of hematologic malignancies was revised to reflect advances in the field.⁶ In-depth discussion of each of the subclassifications is beyond the scope of this book, so only the most common subtypes of acute lymphoblastic leukemia and acute myeloid leukemia are detailed here.

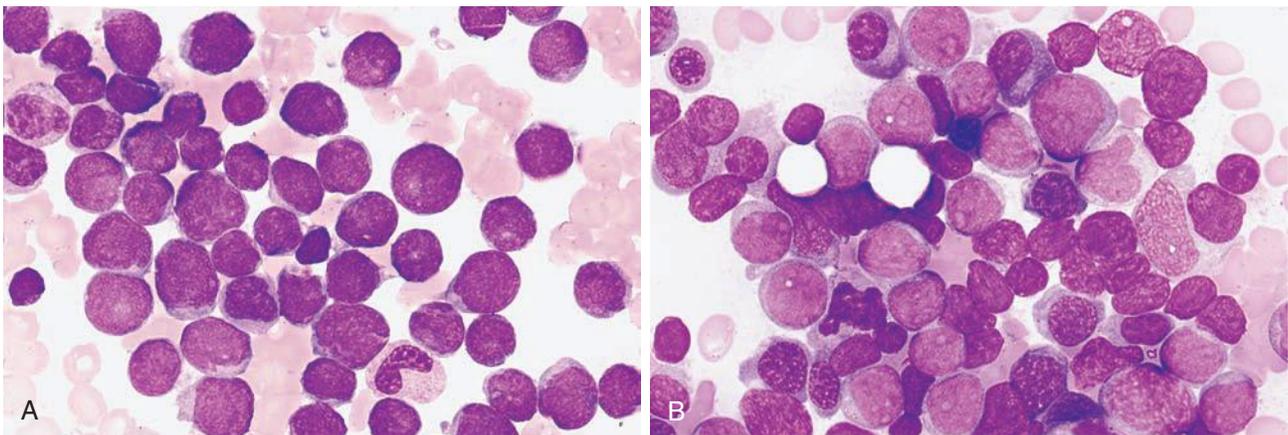


Figure 35-2 **A**, Lymphoblasts (bone marrow, Wright stain, $\times 500$). Cells have a diameter two to three times the normal lymphocyte diameter, scant blue cytoplasm, coarse chromatin, deeper staining than myeloblasts, and inconspicuous nucleoli. **B**, Myeloblasts (bone marrow, Wright stain, $\times 500$). Cells have a diameter three to five times the lymphocyte diameter, moderate gray cytoplasm, uniform fine chromatin, two or more prominent nucleoli, and possibly Auer rods.

ACUTE LYMPHOBLASTIC LEUKEMIA

Acute lymphoblastic leukemia (ALL) is primarily a disease of childhood and adolescence, accounting for 25% of childhood cancers and up to 75% of childhood leukemia.⁷ The peak incidence of ALL in children is between 2 and 5 years of age.⁸ Although ALL is rare in adults, risk increases with age; most adult patients are older than 50 years of age. The subtype of ALL is an important prognostic indicator for survival.⁶ Adults have a poorer outlook: 80% to 90% experience complete remission, but the cure rate is less than 40%.^{9,10}

Patients with B cell ALL typically present with fatigue (caused by anemia), fever (caused by neutropenia and infection), and mucocutaneous bleeding (caused by thrombocytopenia). Lymphadenopathy, including enlargement, is often a symptom.¹¹ Enlargement of the spleen (splenomegaly) and of the liver (hepatomegaly) may be seen. Bone pain often results from intramedullary growth of leukemic cells.¹¹ Eventual infiltration of malignant cells into the meninges, testes, or ovaries occurs frequently, and lymphoblasts can be found in the cerebrospinal fluid.¹²

In T cell ALL, there may be a large mass in the mediastinum leading to compromise of regional anatomic structures. Similar to B-ALL, T-ALL may present with anemia, thrombocytopenia, organomegaly, and bone pain, although the degree of leukopenia is often less severe.¹³

World Health Organization Classification

B lymphoblastic leukemia/lymphoma (B-ALL) is subdivided into seven subtypes that are associated with recurrent cytogenetic abnormalities.¹⁴ These entities are linked with unique clinical, phenotypic, or prognostic features (Box 35-1). Cases of B cell ALL that do not exhibit the specific genetic abnormalities are classified as B lymphoblastic leukemia/lymphoma, not otherwise specified. Although 50% to 70% of patients with T lymphoblastic leukemia/lymphoma have abnormal gene rearrangements, none of the abnormalities is clearly associated with specific biologic features, and thus T-ALL is not further subdivided clinically.¹⁴

Morphology

Lymphoblasts vary in size but fall into two morphologic types. The most common type seen is a small lymphoblast (1.0 to 2.5 times the size of a normal lymphocyte) with scant blue cytoplasm and indistinct nucleoli (Figure 35-2); the second type of lymphoblast is larger (two to three times the size of a lymphocyte) with prominent nucleoli and nuclear membrane irregularities (Figure 35-3).¹³ These cells may be confused with the blasts of acute myeloid leukemia (AML).

Prognosis

Prognosis in ALL has improved dramatically over the past decades as a result of improvement in algorithms for treatment.¹³ The prognosis for ALL depends on age at the time of diagnosis, lymphoblast load (tumor burden), immunophenotype, and

BOX 35-1 B Lymphoblastic Leukemia/Lymphoma with Recurrent Genetic Abnormalities (2008 World Health Organization Classification)

B lymphoblastic leukemia/lymphoma with t(9;22)(q34;q11.2); *BCR-ABL1*
 B lymphoblastic leukemia/lymphoma with t(v;11q23); *MLL* rearranged
 B lymphoblastic leukemia/lymphoma with t(12;21)(p13;q22); *TEL-AML1 (ETV6-RUNX1)*
 B lymphoblastic leukemia/lymphoma with hyperdiploidy
 B lymphoblastic leukemia/lymphoma with hypodiploidy
 B lymphoblastic leukemia/lymphoma with t(5;14)(q31;q32); *IL3-IGH*
 B lymphoblastic leukemia/lymphoma with t(1;19)(q23;p13.3); *E2A-PBX1 (TCF3-PBX1)*

From Swerdlow SH, Campo E, Harris NL, et al, editors: *WHO classification of tumours of haematopoietic and lymphoid tissues*, ed 4, Lyon, France, 2008, IARC Press.

genetic abnormalities. Children rather than infants or teens do the best. Chromosomal translocations are the strongest predictor of adverse treatment outcomes for children and adults. Peripheral blood lymphoblast counts greater than 20 to 30 × 10⁹/L, hepatosplenomegaly, and lymphadenopathy all are associated with worse outcome. The effects of other variables previously associated with a poorer prognosis, such as sex and ethnic group, have been eliminated when patients have been given equal access to treatment in trials carried out at a single institution.¹⁵

Immunophenotyping

Although morphology is the first tool used to distinguish ALL from AML, immunophenotyping and genetic analysis are the most reliable indicators of a cell's origin. Because both B and T cells are derived from lymphoid progenitors, both usually express CD34, terminal deoxynucleotidyl transferase (TdT),

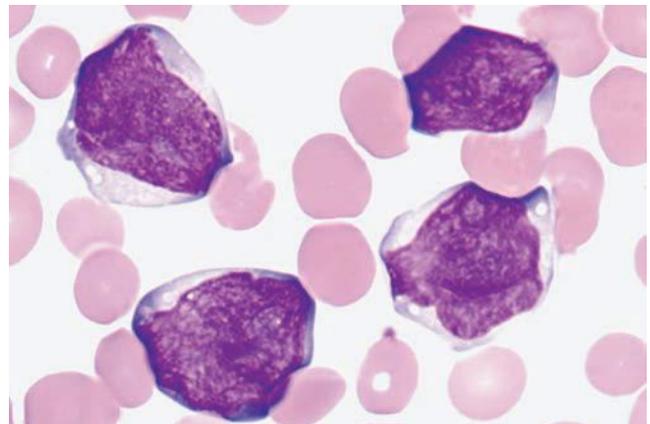


Figure 35-3 Acute lymphoblastic leukemia. Large lymphoblast with prominent nucleoli and membrane irregularities (peripheral blood, ×1000). (From Carr JH, Rodak BF: *Clinical hematology atlas*, ed 4, St. Louis, 2013, Saunders.)

and HLA-DR. Four types of ALL have been identified by immunologic methods: early B-ALL (pro-B, or pre-pre-B), intermediate (common) B-ALL, pre-B-ALL, and T-ALL (Table 35-1). B-ALL is characterized by specific B cell antigens that are expressed at different stages of B cell development. In general, B cells express CD19, CD20, CD22, CD24, C79a, CD10, cytoplasmic μ , and PAX-5 (B cell specific activator protein). The degree of differentiation of B-lineage lymphoblasts often correlates with genetics and plays an important role in treatment decisions.^{6,13,16} In the earliest stage of differentiation (pre-pre B or pro-B), blasts express CD34, CD19, cytoplasmic CD22, and TdT. The incidence of pro-B ALL is about 5% in children and 11% in adults. In intermediate or *common* B-ALL, CD10 is expressed. The most mature B-ALL is called *pre-B-ALL*, in which CD34 is typically negative, but there is characteristic expression of cytoplasmic μ heavy chain. Pre-B-ALL accounts for 15% of childhood cases and 10% of adult B-ALL.¹⁷

T-ALL is seen most often in teenaged males with a mediastinal mass, elevated peripheral blast counts, meningeal involvement, and infiltration of extra marrow sites.^{18,19} The common T cell markers CD2, CD3, CD4, CD5, CD7, and CD8 are usually present. Most cases express TdT. A distinct subtype of T-ALL, ETP-ALL (early T cell precursor ALL), contains a characteristic immunophenotype (CD8-, CD5dim) and has a poor response to chemotherapy, low rates of remission, and overall poor survival.^{20,21}

Genetic and Molecular Findings

Cytogenetic abnormalities are seen in the majority of B and T cell ALL, which produce changes that affect normal B and T cell development and underlie the pathogenesis of these neoplasms. A majority of T-ALL have been shown to have gain-of-function mutations involving the *NOTCH1* gene, which alters the Notch receptor signaling pathway responsible for normal T cell development.²²

In T-ALL, however, the cytogenetic alterations show less specificity and less correlation with prognosis and treatment outcome than in B-ALL. B lymphoblastic leukemia/lymphoma with the t(9;22)(q34;q11.2);*BCR-ABL1* mutation (Philadelphia chromosome-positive ALL) has the worst prognosis among ALLs. It is more common in adults than in children. Imatinib, which has shown success in treating chronic myelogenous leukemia, has improved survival (Chapter 33).

TABLE 35-1 Immunophenotypic Characteristics of Acute Lymphoblastic Leukemia

ALL Subtype	Immunophenotype
Early (pro/pre-pre) B-ALL	CD34, CD19, cytoplasmic CD22, TdT
Intermediate (common) B-ALL	CD34, CD19, CD10, cytoplasmic CD22, TdT
Pre-B-ALL	CD34, CD19, cytoplasmic CD22, cytoplasmic μ , TdT (variable)
T-ALL	CD2, CD3, CD4, CD5, CD7, CD8, TdT

B lymphoblastic leukemia/lymphoma with t(v;11q23);*MLL* rearranged is more common in very young infants, and the translocation may even occur in utero.²³ This leukemia has a very poor prognosis. About 25% of childhood ALL cases show a t(12;21)(p13;q22);*ETV6-RUNX1* translocation and appear to derive from a B cell progenitor rather than the hematopoietic stem cell.²⁴ This translocation is rare in adults. In children, it carries an excellent prognosis, with a cure rate of over 90%. Hyperdiploidy in B lymphoblastic leukemia/lymphoma is common in childhood B-ALL, accounting for 25% of cases, but it is much less common in adults. This genotype is associated with a very favorable prognosis in children. Conversely, hypodiploidy (less than 46 chromosomes) conveys a poor prognosis in both children and adults.

ACUTE MYELOID LEUKEMIA

AML is the most common type of leukemia in adults, and the incidence increases with age. AML is less common in children. The French-American-British (FAB) classification of AML was based on morphology and cytochemistry; the WHO classification relies heavily on cytogenetics and molecular characterization (Chapters 30 and 31).^{6,14}

Clinical Presentation

The clinical presentation of AML is nonspecific but reflects decreased production of normal bone marrow elements. Most patients with AML have a total WBC count between 5 and 30 $\times 10^9/L$, although the WBC count may range from 1 to 200 $\times 10^9/L$. Myeloblasts are present in the peripheral blood in 90% of patients. Anemia, thrombocytopenia, and neutropenia give rise to the clinical findings of pallor, fatigue, fever, bruising, and bleeding. In addition, disseminated intravascular coagulation and other bleeding abnormalities can be significant.²⁵ Infiltration of malignant cells into the gums and other mucosal sites and skin also can be seen.

Splenomegaly is seen in half of AML patients, but lymph node enlargement is rare. Cerebrospinal fluid involvement in AML is rare and does not seem to be as ominous a sign as in ALL. Patients with AML tend to have few symptoms related to the central nervous system, even when it is infiltrated by blasts.

Common abnormalities in laboratory test results include hyperuricemia (caused by increased cellular turnover), hyperphosphatemia (due to cell lysis), and hypocalcemia (the latter two are also involved in progressive bone destruction). Hypokalemia is also common at presentation. During induction chemotherapy, especially when the WBC is quite elevated, tumor lysis syndrome may occur. Tumor lysis syndrome is a group of metabolic complications that can occur in patients with malignancy, most notably lymphomas and leukemias, with and without treatment of the malignancy. These complications are caused by the breakdown products of dying cancer

cells, which in turn cause acute uric acid nephropathy and renal failure. Tumor lysis syndrome is characterized by hyperkalemia, hyperphosphatemia, hyperuricemia and hyperuricosuria, and hypocalcemia.²⁶ The hyperkalemia alone can be life-threatening. Aggressive prophylactic measures to prevent or reduce the clinical manifestations of tumor lysis syndrome are critical.²⁷

Subtypes of Acute Myeloid Leukemia and Related Precursor Neoplasms

Laboratory diagnosis of AML begins with a complete blood count, peripheral blood film examination, and bone marrow aspirate and biopsy specimen examination. The total WBC count may be normal, increased, or decreased; anemia is usually present, along with significant thrombocytopenia. The bone marrow is usually hypercellular, and greater than 20% of cells typically are marrow blasts, although if certain genetic abnormalities are present, the 20% blast threshold is not necessary for the diagnosis of AML.⁶ Each category is discussed, and a summary of the classification is presented in [Box 35-2](#).

The 2008 WHO classification for myeloid malignancies has categorized AMLs with recurrent cytogenetic abnormalities into subgroups based on the primary cytogenetic aberrations ([Box 35-3](#)).^{6,14}

BOX 35-2 Acute Myeloid Leukemia and Related Precursor Neoplasms (2008 World Health Organization Classification)

Acute myeloid leukemia with recurrent genetic abnormalities
 Acute myeloid leukemia with myelodysplasia-related changes
 Therapy-related myeloid neoplasms
 Acute myeloid leukemia, not otherwise specified
 Myeloid sarcoma
 Myeloid proliferations related to Down syndrome
 Blastic plasmacytoid dendritic cell neoplasm

From Swerdlow SH, Campo E, Harris NL, et al, editors: *WHO classification of tumours of haematopoietic and lymphoid tissues*, ed 4, Lyon, France, 2008, IARC Press.

BOX 35-3 Acute Myeloid Leukemia with Recurrent Genetic Abnormalities (2008 World Health Organization Classification)

AML with t(8;21)(q22;q22); *RUNX1-RUNX1T1*
 AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22); *CBFB-MYH11*
 APL with t(15;17)(q22;q12); *PML-RARA*
 AML with t(9;11)(p22;q23); *MLL3-MLL*
 AML with t(6;9)(p23;q34); *DEK-NUP214*
 AML with inv(3)(q21q26.2) or t(3;3)(q21;q26.2); *RPN1-EV1*
 AML with t(1;22)(p13;q13) *RBM15-MKL1*
 Provisional entity: AML with mutated *NPM1*
 Provisional entity: AML with mutated *CEBPA*

From Swerdlow SH, Campo E, Harris NL, et al, editors: *WHO classification of tumours of haematopoietic and lymphoid tissues*, ed 4, Lyon, France, 2008, IARC Press.

AML with Recurrent Genetic Abnormalities

Acute Myeloid Leukemia with t(8;21)(q22;q22);*RUNX1/RUNX1T1*. The t(8;21)(q22;q22);*RUNX1/RUNX1T1* mutation is found in about 5% of AML cases. Seen predominantly in children and young adults, AML with this translocation has myeloblasts with dysplastic granular cytoplasm, Auer rods, and some maturation ([Figure 35-4](#)), similar to the FAB M2 classification (see later in chapter). Various anomalies, such as pseudo-Pelger-Huët cells and hypogranulation, can be seen. Eosinophilia is possible. Prognosis is generally favorable but may be negatively impacted if unfavorable additional abnormalities, such as monosomy 7, occur.²⁸ The diagnosis of this subtype is based on the genetic abnormality, regardless of blast count.⁶

Acute Myeloid Leukemia with inv(16)(p13.1q22) or t(16;16)(p13.1;q22);*CBFB-MYH11*. Accounting for approximately 5% to 8% of all AML cases, core-binding factor (CBF) AML occurs at all ages, but it is found predominantly in younger patients.⁶ The genetic aberration is sufficient for diagnosis regardless of blast count.^{6,29} Myeloblasts, monoblasts, and promyelocytes are seen in the peripheral blood and bone marrow. In the bone marrow there may be eosinophilia with dysplastic changes ([Figure 35-5](#)). The incidence of extramedullary disease is higher than in most types of AML, and the central nervous system is a common site for relapse.^{6,29} The remission rate is good, but only one half of patients are cured.²⁹

Acute Myeloid Leukemia with t(15;17)(q22;q12);*PML-RARA*. Also known as *acute promyelocytic leukemia* (APL), AML with the t(15;17)(q22;q12);*PML-RARA* mutation comprises 5% to 10% of AML cases. It occurs in all age groups but is seen most commonly in young adults. This disorder is characterized by a differentiation block at the promyelocytic stage. The abnormal promyelocytes are considered to be comparable to blasts for the purpose of diagnosis. Detection of the 15;17 translocation is sufficient for diagnosis regardless of blast count.^{6,28} Characteristic of this presentation are the

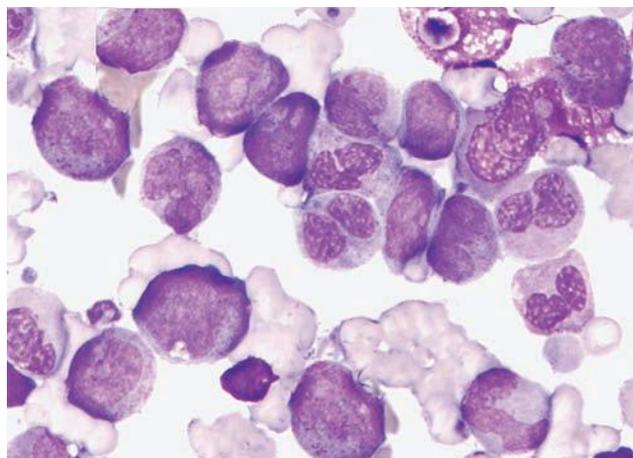


Figure 35-4 Acute myeloid leukemia with t(8;21). Myeloblasts with granular cytoplasm and some maturation (bone marrow, $\times 500$). (From Carr JH, Rodak BF: *Clinical hematology atlas*, ed 4, St. Louis, 2013, Saunders.)

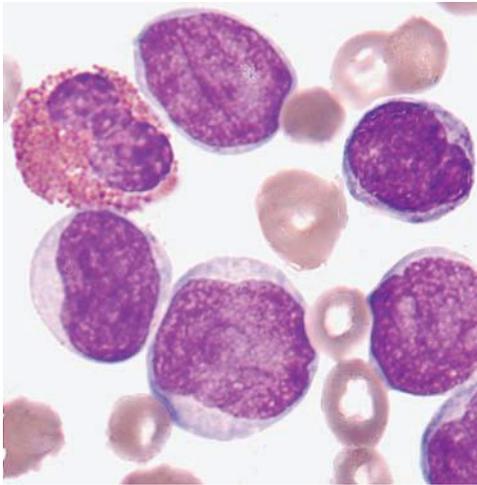


Figure 35-5 Acute myeloid leukemia with $inv(16)$. There is an increase in myeloid and monocytic lines. Eosinophilia may also be present (peripheral blood, $\times 1000$). (From Carr JH, Rodak BF: *Clinical hematology atlas*, ed 4, St. Louis, 2013, Saunders.)

abnormal hypergranular promyelocytes, some with Auer rods (Figure 35-6). When promyelocytes release primary granule contents, their procoagulant activity initiates disseminated intravascular coagulation; however, thromboembolic events may occur at presentation and during treatment.³⁰ In one variant of APL, the granules are so small that because of the limits of light microscopy, the cells give the appearance of having no granules. This microgranular variant, accounting for 30% to 40% of APL cases, may be confused with other presentations of AML, but the presence of occasional Auer rods, the “butterfly” or “coin-on-coin” nucleus, and the clinical presentation are clues. The treatment of APL is significantly different from all other types of acute myeloid leukemia, and it is therefore important to arrive at an accurate diagnosis. Treatment includes all-*trans*-retinoic acid (ATRA) and arsenic trioxide.³¹ ATRA is a vitamin A analogue and induces differentiation of the malignant promyelocytes. In adults who achieve a complete remission, the prognosis is better than for any other type of AML.²⁸ There are a few variants in *RARA* translocations that confer a poor diagnosis because the cells do not respond to ATRA therapy.^{6,14}

Acute Myeloid Leukemia with $t(9;11)(p22;q23);MLL3-MLL$. AML with $t(9;11)(p22;q23);MLL3-MLL$ represents a specific subgroup of the previous classification of AML with 11q23 abnormalities, and AMLs with other *MLL* abnormalities should not be placed in this group.¹⁴ AML with $t(9;11)$ is a rare leukemia (6% of AML cases) that presents with an increase in monoblasts and immature monocytes (Figure 35-7). The blasts are large with abundant cytoplasm and fine nuclear chromatin. The cells may have motility, with pseudopodia seen frequently. Granules and vacuoles can be observed in the blasts. Typically this disease occurs in children and may be associated with gingival and skin involvement and/or disseminated intravascular coagulation. The prognosis is intermediate to poor.²⁸

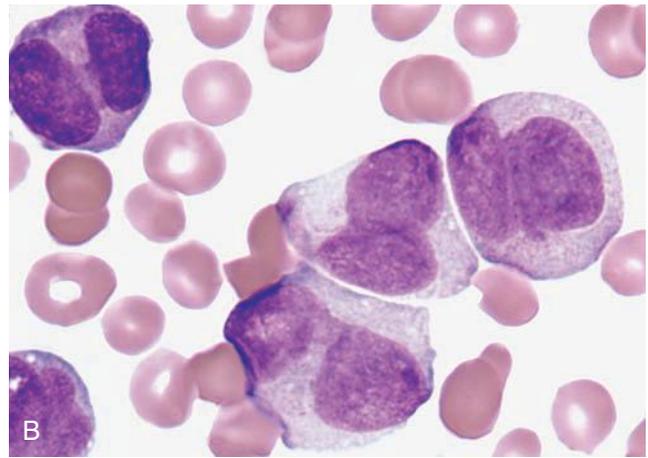
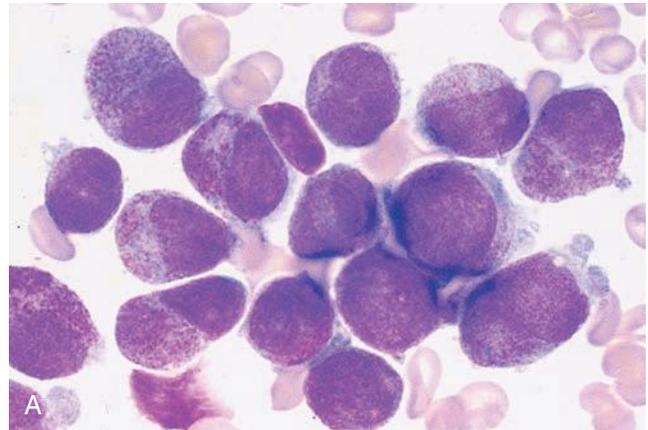


Figure 35-6 Acute myeloid leukemia with $t(15;17)$, or promyelocytic leukemia. **A**, Low-power view of the more common hypergranular variant (peripheral blood, $\times 500$). **B**, Oil immersion view of the microgranular variant showing bilobed nuclear features (peripheral blood, $\times 1000$). (**B** from Carr JH, Rodak BF: *Clinical hematology atlas*, ed 4, St. Louis, 2013, Saunders.)

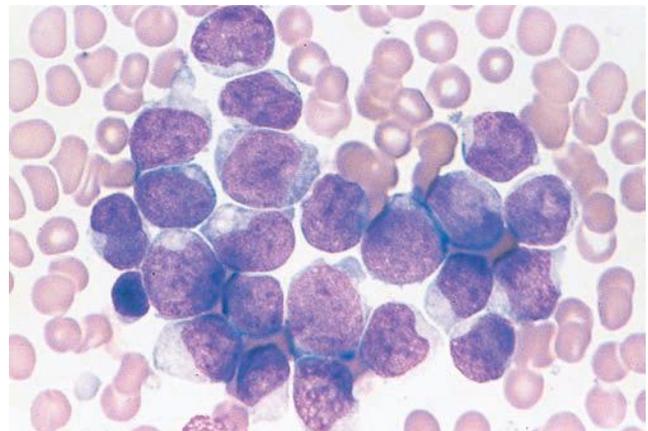


Figure 35-7 Acute myeloid leukemia with $t(9;11)$ abnormalities. Both monoblasts and immature monocytes are increased (bone marrow, $\times 500$).

Acute Myeloid Leukemia with $t(6;9)(p23;q34);DEK-NUP214$, Acute Myeloid Leukemia with $inv(3)(q21q26.2)$ or $t(3;3)(q21;q26.2);RPN1-EV11$, and Acute Myeloid Leukemia (Megakaryoblastic) with $t(1;22)(p13;q13);RBM15-MKL1$. These are rare leukemias included in the 2008 WHO classification. Detailed description of these entities is beyond the scope of this chapter.

Acute Myeloid Leukemia with Myelodysplasia-Related Changes

AML with myelodysplasia affects primarily older adults and has a poor prognosis. This subcategory of AML with myelodysplasia-related changes incorporates leukemias with at least 20% blasts, multilineage dysplasia, a history of MDS or MDS/myeloproliferative neoplasm (MPN), or a specific MDS-associated cytogenetic abnormality and the absence of AML with recurrent genetic abnormalities. Significant dysplastic morphology includes pancytopenia with neutrophil hypogranulation or hypergranulation, pseudo-Pelger-Huët cells, and unusually segmented nuclei. Erythrocyte precursors have vacuoles, karyorrhexis, megakaryoblastoid features, and ringed sideroblasts. There may be dysplastic micromegakaryocytes and dysplastic megakaryocytes. Genetic findings are similar to those found in MDS, with complex karyotypes and $-7/\text{del}(7q)$ and $-5/\text{del}(5q)$ being the most common.^{6,33}

Therapy-Related Myeloid Neoplasms

Treatment with alkylating agents, radiation, or topoisomerase II inhibitors has been associated with the development of a secondary AML, MDS, or MDS/MPN.^{6,28,34,35} These therapy-related neoplasms account for 10% to 20% of AMLs, MDSs, and MDSs/MPNs. Generally these disorders occur following treatment for a prior malignancy, but they have also been associated with intensive treatment of patients with nonmalignant disorders requiring cytotoxic therapy.^{6,34,36} Therapy-related myeloid neoplasms are similar in morphology to AML with myelodysplasia, monocytic/monoblastic leukemia, or AML with maturation, and the prognosis is generally poor, although therapy-related neoplasms with the $t(15;17)$ and $\text{inv}(16)$ mutations behave more like the de novo counterparts.^{6,28}

Acute Myeloid Leukemia, Not Otherwise Specified

Because the leukemias in the “not otherwise specified” category do not fit easily into the WHO subtypes described earlier, they are grouped according to morphology, flow cytometric phenotyping (Chapter 32), and limited cytochemical reactions, as in the FAB classification. The FAB classification was based on the cell of origin, degree of maturity, cytochemical reactions, and limited cytogenetic features (Table 35-2).^{37,38} A blast percentage of at least 20% in the peripheral blood or bone marrow is required for diagnosis. This category accounts for about 25% of all AML, but as more genetic subgroups are recognized, the number in this group will diminish.¹⁴

Acute Myeloid Leukemia with Minimal Differentiation.

The blasts in AML with minimal differentiation are CD13⁺, CD33⁺, CD34⁺, and CD117⁺ (Figure 35-8).^{6,39} Auer rods typically are absent, and there is no clear evidence of cellular maturation. The cells yield negative results with the cytochemical stains myeloperoxidase and Sudan black B. These cases account for less than 5% of AML, and patients are generally either infants or older adults.

Acute Myeloid Leukemia without Maturation. Closely aligned with the blasts in minimally differentiated AML, the

TABLE 35-2 French-American-British Classification of the Acute Myeloid Leukemias

Subtype	Description
M0	Acute myeloid leukemia, minimally differentiated
M1	Acute myeloid leukemia without maturation
M2	Acute myeloid leukemia with maturation
M3	Acute promyelocytic leukemia
M4	Acute myelomonocytic leukemia
M4eo	Acute myelomonocytic leukemia with eosinophilia
M5a	Acute monocytic leukemia, poorly differentiated
M5b	Acute monocytic leukemia, well differentiated
M6	Acute erythroleukemia
M7	Acute megakaryocytic leukemia

Data from Bennett JM, Catovsky D, Daniel MT, et al: Proposals for the classification of the acute leukemias. French-American-British (FAB) co-operative group, *Br J Haematol* 33:451-458, 1976; and Bennett JM, Catovsky D, Daniel MT, et al: Proposed revised criteria for the classification of acute myeloid leukemia. A report of the French-American-British Cooperative Group, *Ann Intern Med* 103:620-625, 1985.

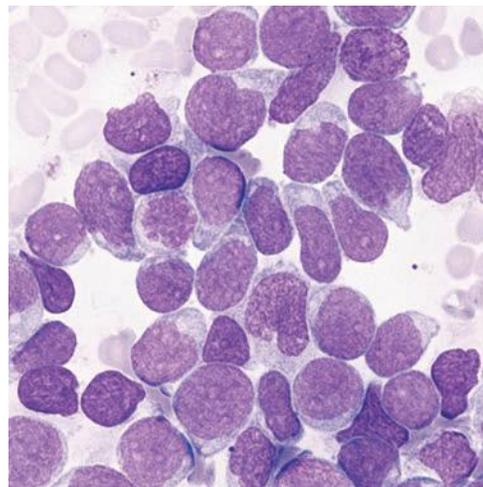


Figure 35-8 Acute myeloid leukemia, minimally differentiated (French-American-British classification M0). Blasts lack myeloid morphologic features and yield negative results with myeloperoxidase and Sudan black B staining. Auer rods are not seen. CD34 is frequently present (bone marrow, $\times 500$). (From Carr JH, Rodak BF: *Clinical hematology atlas*, ed 4, St. Louis, 2013, Saunders.)

blasts in AML without maturation are also CD13⁺, CD33⁺, and CD117⁺, and CD34 is present in about 70% of cases (Figure 35-9).⁶ Blasts may comprise 90% of nonerythroid cells in the bone marrow, and fewer than 10% of the leukocytes show maturation to the promyelocyte stage or beyond. Blasts have Auer rods and usually give positive results with myeloperoxidase or Sudan black B stains.^{6,28}

Acute Myeloid Leukemia with Maturation. AML with maturation is a common variant that presents with greater than 20% blasts, at least 10% maturing cells of neutrophil lineage (Figure 35-10), and fewer than 20% precursors with monocytic lineage. Auer rods and other aspects of dysplasia are present.⁶

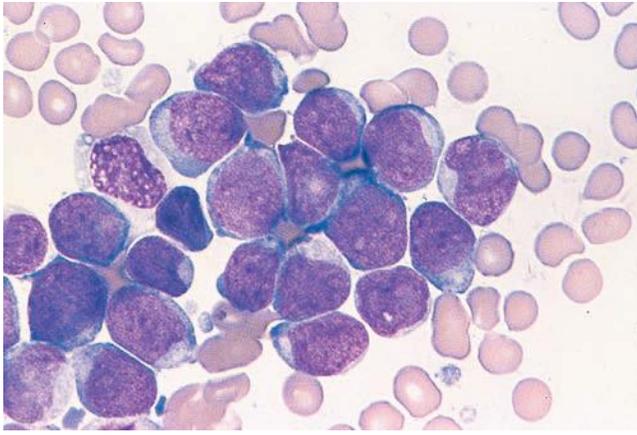


Figure 35-9 Acute myeloid leukemia without maturation (French-American-British classification M1). Blasts constitute 90% of the nonerythroid cells; there is less than 10% maturation of the granulocytic series beyond the promyelocyte stage (bone marrow, $\times 500$).

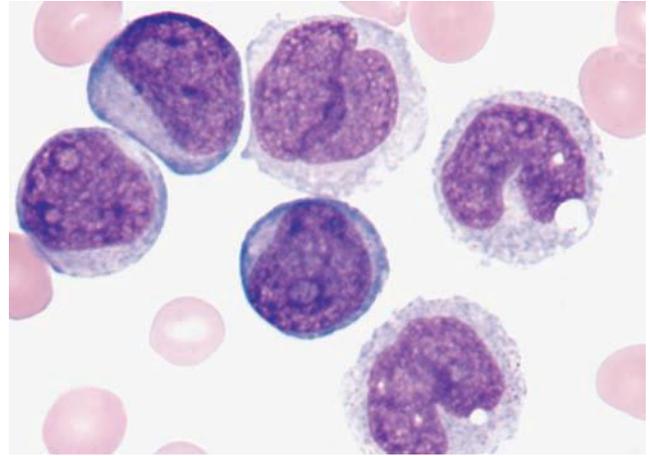


Figure 35-11 Acute myelomonocytic leukemia. Both myeloid and monocytic cells are present. Monocytic cells comprise at least 20% of all marrow cells, with monoblasts and promonocytes present (peripheral blood, $\times 1000$). (From Carr JH, Rodak BF: *Clinical hematology atlas*, ed 4, St. Louis, 2013, Saunders.)

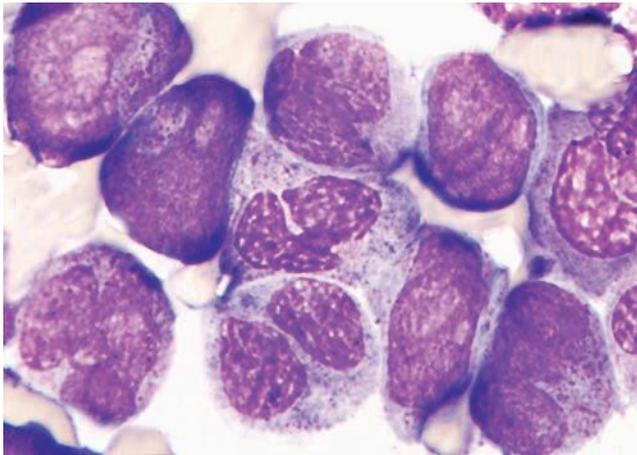


Figure 35-10 Acute myeloid leukemia with maturation. Blasts constitute 20% or more of the nucleated cells of the bone marrow, and there is maturation beyond the promyelocyte stage in more than 10% of the nonerythroid cells (bone marrow, $\times 1000$).

Acute Myelomonocytic Leukemia. Acute myelomonocytic leukemia is characterized by a significantly elevated WBC count and the presence of myeloid and monocytoid cells in the peripheral blood and bone marrow (Figure 35-11). Monocytic cells (monoblasts and promonocytes) constitute at least 20% of all marrow cells. The monoblasts are large with abundant cytoplasm containing small granules and pseudopodia. The nucleus is large and immature and may contain multiple nucleoli. Promonocytes also are present and may have contorted nuclei. The cells are positive for the myeloid antigens CD13 and CD33 and the monocytic antigens CD14, CD4, CD11b, CD11c, CD64, and CD36. Nonspecific cytogenetic changes are found in most cases.⁶

Acute Monoblastic and Monocytic Leukemias. In these leukemias, which are divided into monoblastic and monocytic based on the degree of maturity of the monocytic cells present in the marrow and peripheral blood, more than

80% of the marrow cells are of monocytic origin. These cells are CD14⁺, CD4⁺, CD11b⁺, CD11c⁺, CD36⁺, CD64⁺, and CD68⁺. Blasts are large with abundant, often agranular cytoplasm and large prominent nucleoli (Figure 35-12, A). When some evidence of maturation is present, the cells are called *promonocytes*. Promonocytes in monocytic leukemias with differentiation are considered to be blast equivalents (Figure 35-12, B). Nonspecific esterase testing usually yields positive results. Acute monoblastic/monocytic leukemia comprises fewer than 5% of cases of AML and is most common in younger individuals. Extramedullary involvement, including cutaneous and gingival infiltration, and bleeding disorders are common. Nonspecific cytogenetic abnormalities are seen in most cases.^{6,40}

Acute Erythroid Leukemia. According to the WHO classification, there are two subtypes of acute erythroid leukemia, based on the presence of a significant component of myeloblasts. The first is *acute erythroleukemia* (erythroid/myeloid), in which 50% or more of nucleated bone marrow cells are normoblasts and greater than 20% are myeloblasts. In the FAB classification, this subtype was known as M6.

The second type is *pure erythroid leukemia*. In this type, 50% or more nucleated cells are pronormoblasts and 30% or more are basophilic normoblasts. Together, these two erythroid components comprise more than 80% of the bone marrow. The myeloblast component is not significant. Complex rearrangements and hypodiploid chromosome number are common. Chromosomes 5 and 7 are frequently affected.⁶

The red blood cell (RBC) precursors have significant dysplastic features, such as multinucleation, megaloblastoid asynchrony, and vacuolization. The nucleated RBCs in the peripheral blood may account for more than 50% of the total number of nucleated cells. Ringed sideroblasts, Howell-Jolly bodies, and other inclusions may be present (Figure 35-13). Abnormal megakaryocytes may be seen. Both types of erythroid leukemia have an aggressive and rapid clinical course.⁶

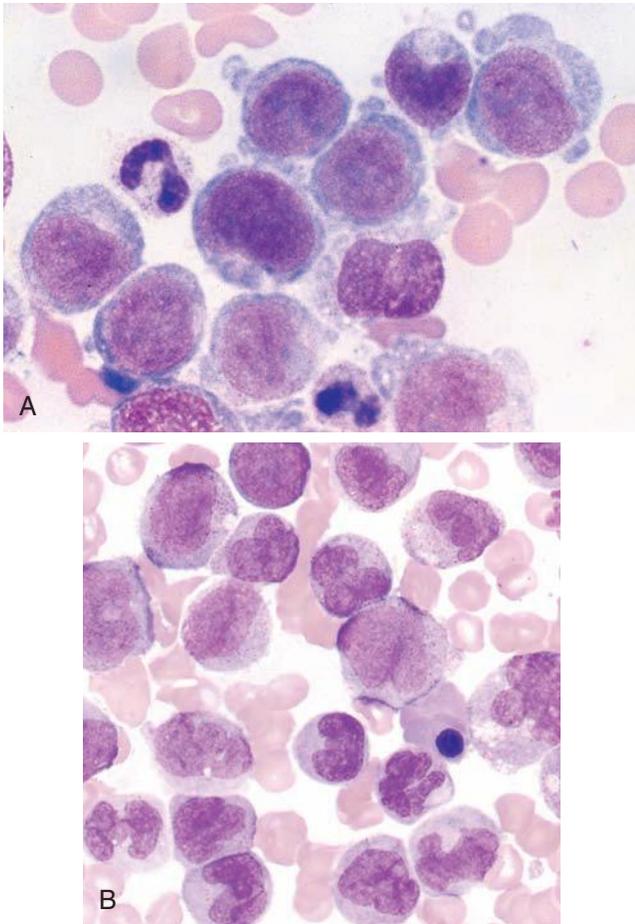


Figure 35-12 **A**, Acute monoblastic leukemia. More than 80% of the bone marrow cells are of monocytic origin (bone marrow, $\times 500$). **B**, Acute monoblastic leukemia with promonocytes. Promonocytes are considered blast equivalents. (**B** from Carr JH, Rodak BF: *Clinical hematology atlas*, ed 4, St. Louis, 2013, Saunders.)

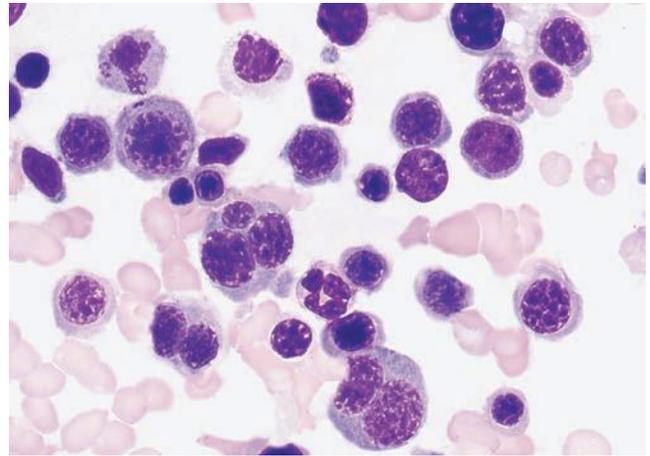


Figure 35-13 Acute erythroid leukemia. Erythroid precursors showing dysplastic features, including multinucleation and megaloblastic asynchrony (bone marrow, $\times 500$). (From Carr JH, Rodak BF: *Clinical hematology atlas*, ed 4, St. Louis, 2013, Saunders.)

Acute Megakaryoblastic Leukemia. Patients with acute megakaryoblastic leukemia usually have cytopenias, although some may have thrombocytosis. Dysplastic features are often present in all cell lines. Diagnosis requires the presence of at least 20% blasts, of which at least 50% must be of megakaryocyte origin. This category excludes AML with MDS-related changes and Down syndrome-related cases, as well as those with recurrent genetic abnormalities, as discussed previously.

Megakaryoblast diameters vary from that of a small lymphocyte to three times their size. Chromatin is delicate with prominent nucleoli. Immature megakaryocytes may have light blue cytoplasmic blebs (**Figure 35-14, A**). Megakaryoblasts are identified by immunostaining, employing antibodies specific for cytoplasmic von Willebrand factor or platelet membrane antigens CD41 (glycoprotein IIb), CD42b (glycoprotein Ib) (**Figure 35-14, B**), or CD61 (glycoprotein IIIa).⁶

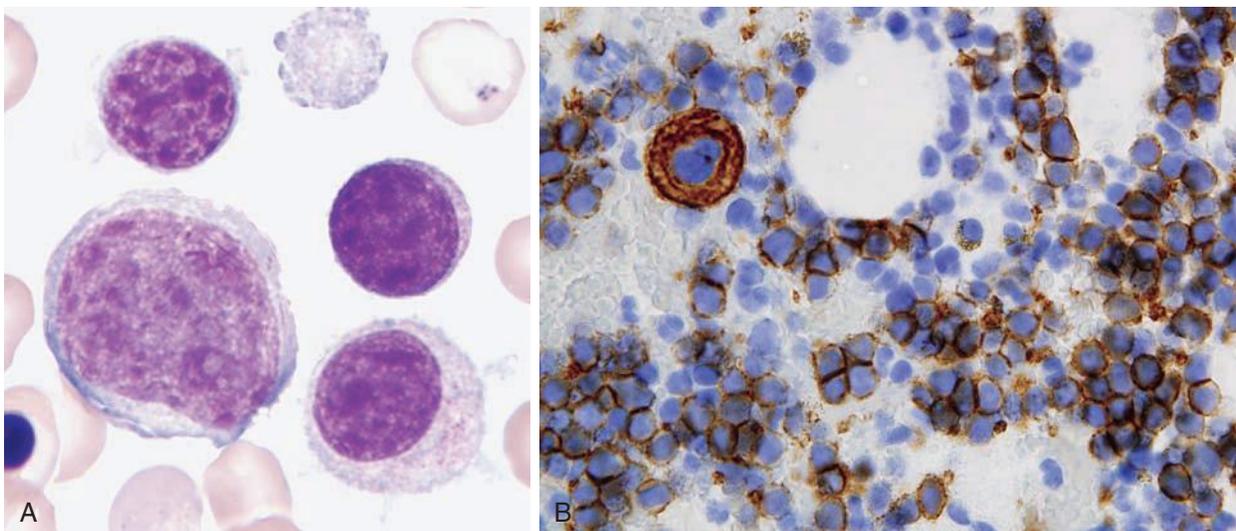


Figure 35-14 Acute megakaryocytic leukemia. **A**, Note heterogeneity of blasts, one small with scant cytoplasm, two with cytoplasmic blebbing, and one quite large (peripheral blood $\times 1000$). **B**, Positive reaction for CD42b (bone marrow, $\times 1000$). (**A** from Carr JH, Rodak BF: *Clinical hematology atlas*, ed 4, St. Louis, 2013, Saunders.)

Myeloid Sarcoma

Myeloid sarcoma refers to extramedullary proliferation of blasts of one or more myeloid lineages that disrupts tissue architecture. Tissue architecture must be effaced for the neoplasm to qualify for this diagnosis.^{6,28}

Myeloid Proliferations Related to Down Syndrome

Unique patterns of malignancy occur in persons with trisomy 21 resulting in Down syndrome. Somatic mutations of the *GATA1* gene have also been detected and are linked to both leukemogenesis and high cure rates.⁴¹ Approximately 10% of newborns with Down syndrome present with transient abnormal myelopoiesis, which is morphologically indistinguishable from AML. Spontaneous remission generally occurs within a few months. Among individuals with Down syndrome, there is a fiftyfold increased incidence of AML during the first 5 years of life compared with individuals without Down syndrome. The leukemia is of megakaryocytic lineage, and young children respond well to chemotherapy, although older children do not fare as well.^{6,41}

Blastic Plasmacytoid Dendritic Cell Neoplasm

Blastic plasmacytoid cell neoplasm is a rare clinically aggressive tumor derived from precursors of plasmacytoid dendritic cells. It presents with skin lesions and may ultimately progress to involve peripheral blood and bone marrow.^{6,14}

ACUTE LEUKEMIAS OF AMBIGUOUS LINEAGE

Acute leukemias of ambiguous lineage (ALALs) include leukemia in which there is no clear evidence of differentiation along a single cell line and are commonly referred to as *acute undifferentiated leukemias* (AULs). Other cases of ALAL that demonstrate a multiplicity of antigens where it is not possible to determine a specific lineage are called *mixed phenotype acute leukemias* (MPALs). The 2008 WHO classification significantly revised the criteria for this designation and is shown in [Box 35-4](#).⁶

BOX 35-4 Classification of Acute Leukemia of Ambiguous Lineage (ALAL) (2008 World Health Organization Classification)

Acute undifferentiated leukemia (AUL)—synonyms: ALAL without differentiation, primitive acute leukemia, stem cell leukemia
 Mixed phenotype acute leukemia (MPAL)—synonyms: biphenotypic acute leukemia, bilineal leukemia, mixed lineage acute leukemia, dual lineage acute leukemia, hybrid acute leukemia:
 MPAL with t(9;22)(q34;q11.2);*BCR-ABL1*
 MPAL with t(v;11q23);*MLL* rearranged
 MPAL B/myeloid, not otherwise specified
 MPAL T/myeloid, not otherwise specified

From Swerdlow SH, Campo E, Harris NL, et al, editors: *WHO classification of tumours of haematopoietic and lymphoid tissues*, ed 4, Lyon, France, 2008, IARC Press.

FUTURE DIRECTIONS IN THE CLASSIFICATION OF ACUTE LEUKEMIAS

A number of recent studies have shown the importance of gene mutations in the pathogenesis of acute leukemias.^{42,43} These mutations also have prognostic importance and are likely to be incorporated into future classifications. Important mutated genes include *KIT*, *FLT3*, *ASXL1*, *TP53*, *CEBPA*, and *NPM1*. AML with mutated *NPM1* and AML with mutated *CEBPA* are provisional entities in the 2008 WHO classification.⁶

CYTOCHEMICAL STAINS AND INTERPRETATIONS

Techniques such as flow cytometry, cytogenetic analysis, and molecular testing are now commonly used in the diagnosis of acute leukemias. However, older techniques such as cytochemical stains still retain their importance. An advantage of cytochemical stains is that they are relatively cheap and can be performed by laboratories throughout the world, including in areas where resources and access to advanced techniques are limited. The cytochemical stains are summarized in [Table 35-3](#).

Myeloperoxidase

Myeloperoxidase (MPO) ([Figures 35-15](#) and [35-16](#)) is an enzyme found in the primary granules of granulocytic cells (neutrophils, eosinophils, and, to a certain extent, monocytes). Lymphocytes do not exhibit MPO activity. This stain is useful for differentiating the blasts of acute myeloid leukemia (AML) from those of acute lymphoblastic leukemia (ALL).

Interpretation

MPO is present in the primary granules of most granulocytic cells, beginning at the promyelocyte stage and continuing throughout maturation. Leukemic myeloblasts are usually positive for MPO. In many cases of the AMLs (without maturation, with maturation, and promyelocytic leukemia), it has been found that more than 80% of the blasts show MPO activity. Auer rods found in leukemic blasts and promyelocytes test strongly MPO positive.

TABLE 35-3 Acute Leukemia Cytochemical Reaction Chart

Condition	MPO	SBB	NASDA	ANBE	ANAE
ALL	—	—	—	—/+ (focal)	—/+ (focal)
AML	+	+	+	—	—
AMML	+	+	+	+	+
AMoL	—	—/+	—	+	+
Megakaryocytic leukemia	—	—	—	—	+

+, Positive reaction; —, negative reaction; —/+, negative or positive reaction; ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; AMML, acute myelomonocytic leukemia; AMoL, acute monocytic leukemia; ANAE, α -naphthyl acetate esterase; ANBE, α -naphthyl butyrate esterase; MPO, myeloperoxidase; NASDA, naphthol AS-D chloroacetate esterase; SBB, Sudan black B.



Figure 35-15 Positive reaction to myeloperoxidase stain in early myeloid cells. Note Auer rod at arrow (bone marrow, $\times 1000$).

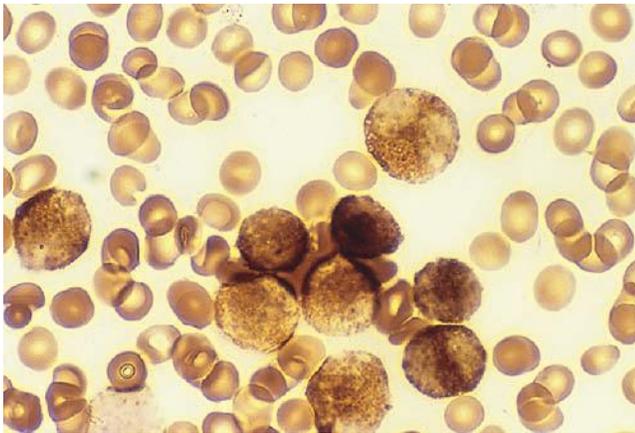


Figure 35-16 Strong positive reaction to myeloperoxidase stain in leukemic promyelocytes from a patient with acute promyelocytic leukemia (bone marrow, $\times 1000$).

In contrast, lymphoblasts in ALL and lymphoid cells are MPO negative. It is important that the reaction only in the blast cells be used as the determining factor for the differentiation of acute leukemias. This is true for MPO and for the other cytochemical stains used in determining cell lineage that are mentioned in this chapter. The fact that maturing granulocytes are MPO positive is normal and has little or no diagnostic significance.

Sudan Black B

SBB staining (Figure 35-17) is another useful technique for the differentiation of AML from ALL. SBB stains cellular lipids. The staining pattern is quite similar to that of MPO; SBB staining is possibly a little more sensitive for the early myeloid cells.

Interpretation

Granulocytes (neutrophils) show a positive reaction to SBB from the myeloblast through the maturation series. The staining becomes more intense as the cell matures as a result of the increase in the numbers of primary and secondary granules.

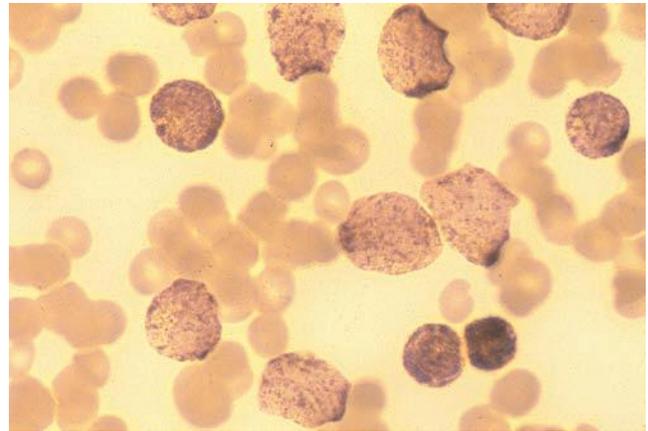


Figure 35-17 Sudan black B reaction. The positivity increases with the maturity of the granulocytic cell (bone marrow, $\times 1000$).

Monocytic cells can demonstrate negative to weakly positive staining due to various changes that occur during differentiation. Lymphoid cells generally do not stain. In ALL, fewer than 3% of the blast cells show a positive reaction.⁴⁴⁻⁴⁶

Esterases

Esterase reactions are used to differentiate myeloblasts and neutrophilic granulocytes from cells of monocytic origin. Nine isoenzymes of esterases are present in leukocytes. Two substrate esters commonly used are α -naphthyl acetate and α -naphthyl butyrate (both nonspecific). Naphthol AS-D chloroacetate (specific) also may be used. "Specific" refers to the fact that only granulocytic cells show staining, whereas nonspecific stains may produce positive results in other cells as well.

Interpretation

Esterase stains can be used to distinguish acute leukemias that are granulocytic from leukemias that are primarily of monocytic origin. When naphthol AS-D chloroacetate is used as a substrate, the reaction is positive in the granulocytic cells and negative to weak in the monocytic cells (Figure 35-18). Chloroacetate esterase is present in the primary granules of neutrophils. Leukemic

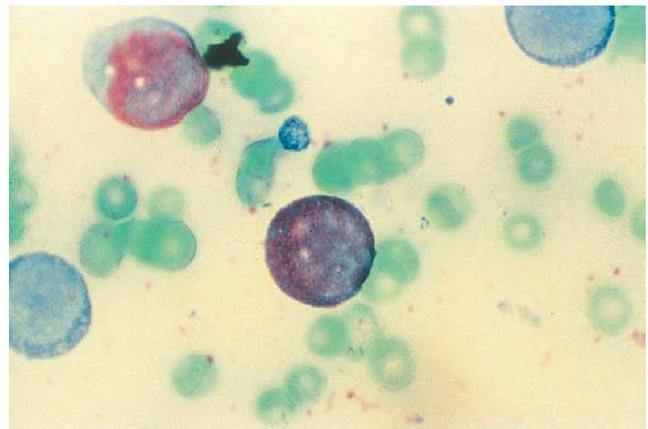


Figure 35-18 Positive reaction to AS-D chloroacetate esterase stain in two granulocytic cells (bone marrow, $\times 1000$).

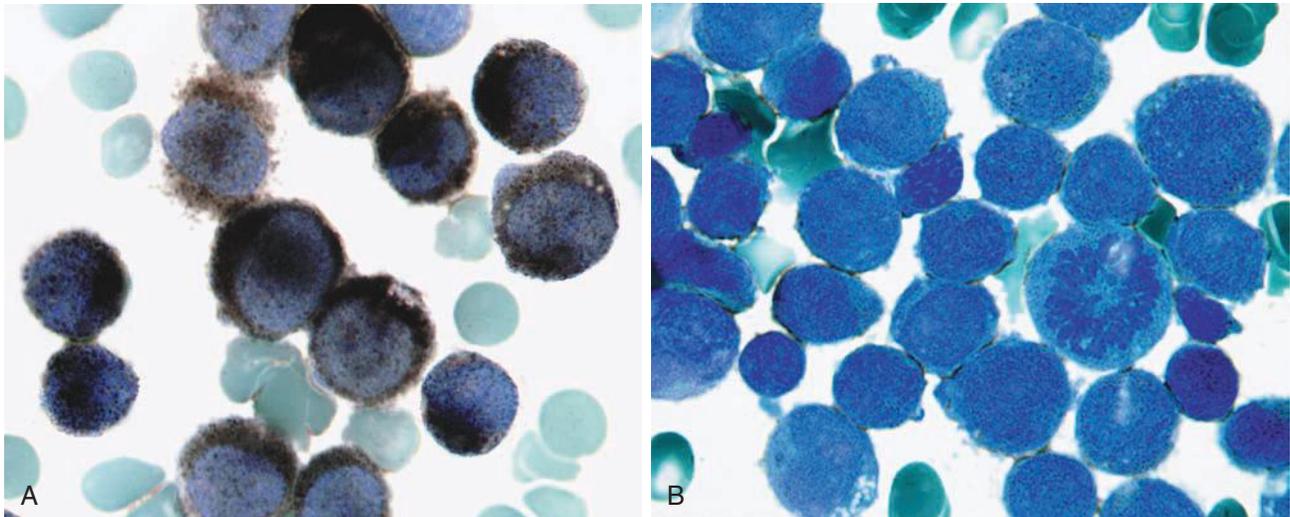


Figure 35-19 **A**, Positive reaction to α -naphthyl acetate esterase stain in monocytes (bone marrow, $\times 1000$). **B**, Same specimen with addition of sodium fluoride. The esterase reaction in the monocytes is inhibited (bone marrow, $\times 1000$).

myeloblasts generally show a positive reaction. Auer rods show positivity as well.

α -Naphthyl acetate, in contrast to naphthol AS-D chloroacetate, reveals strong esterase activity in monocytes that can be inhibited with the addition of sodium fluoride.^{44,46} Granulocytes and lymphoid cells generally show a negative result on nonspecific esterase staining (Figure 35-19).

A diffuse positive α -naphthyl butyrate esterase reaction is seen in monocytes. α -Naphthyl butyrate is less sensitive than α -naphthyl acetate, but it is more specific. Granulocytes and lymphoid cells generally show a negative reaction (Figure 35-20), although a small positive dot may be seen in lymphocytes. In myelomonocytic leukemia, positive AS-D chloroacetate activity and positive α -naphthyl butyrate or α -naphthyl acetate activity should be seen because myeloid and monocytic cells are present. In myelomonocytic leukemia, at least 20% of the cells must show monocytic differentiation that is nonspecific esterase positive and is inhibited by sodium fluoride. In the pure monocytic leukemias, 80% or more of the blasts are nonspecific esterase positive and specific esterase negative.

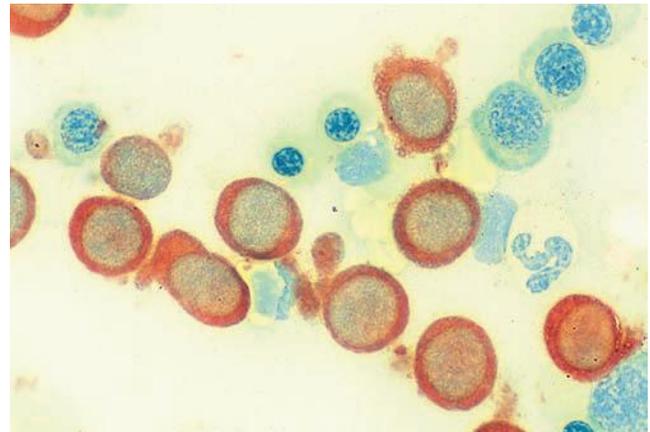


Figure 35-20 α -Naphthyl butyrate esterase positivity in cells of monocytic origin from a patient with acute monoblastic/monocytic leukemia. Note the negativity of myeloid and erythroid precursors (bone marrow, $\times 1000$).

SUMMARY

- The development of leukemia is currently believed to be a stepwise progression of mutations, or “multiple-hits,” involving mutations that give leukemic stem cells a proliferative advantage and also hinder differentiation.
- For most acute leukemias, causes directly related to the development of the malignancy are unknown, but a few exceptions exist. Some known causes include environmental toxins, certain viruses, previous chemotherapy, and familial predisposition.
- There are several classification schemes for leukocyte neoplasia, including the FAB system, based primarily on morphology and cytochemical staining, and the WHO system, which retains some elements of the FAB scheme but emphasizes molecular and cytogenetic changes.
- Only half of patients with ALL have leukocytosis, and many do not have circulating lymphoblasts, but neutropenia, thrombocytopenia, and anemia are usually present.
- In children ALL is a disease in which the “good prognosis” subtypes are associated with a 95% rate of complete remission, but adults with ALL have a poorer outlook.
- Infiltration of malignant cells into the meninges can occur, with lymphoblasts found in the cerebrospinal fluid, testes, and ovaries.

- Prognosis in ALL depends primarily on age at the time of diagnosis, lymphoblast load (tumor burden), and immunophenotype. Chromosomal translocations seem to be the strongest predictor of adverse treatment outcomes for children and adults.
- The t(12;21) marker is found in a significant number of patients with childhood ALL.
- There are two main subtypes of ALL according to the WHO classification system: B lymphoblastic leukemia/lymphoma and T lymphoblastic leukemia/lymphoma.
- Tumor lysis syndrome is an increasingly common complication of treatment, especially in patients with a high tumor burden.
- Although morphology is the first tool in distinguishing ALL from AML, immunophenotyping is often the only reliable indicator of a cell's origin.
- The incidence of AML in adults increases with age.
- The clinical presentation of a patient with AML is nonspecific and reflects the decreased production of normal bone marrow elements, an elevated WBC count, and the presence of myeloblasts. Anemia, thrombocytopenia, and neutropenia give rise to the clinical findings of pallor, fatigue, bruising and bleeding, and fever with infections.
- The classification of AML is complicated by the presence or absence of multiple cell lines defined as “myeloid” in origin, specific cells within these cell lines, and specific karyotype abnormalities.
- Leukemias with ambiguous lineage include leukemias in which there is no clear evidence of differentiation along a single cell line.
- Cytochemical techniques are often used in conjunction with morphologic analysis, immunohistochemical methods, flow cytometry, cytogenetic analysis, and molecular biologic techniques in establishing a diagnosis.
- Cytochemical reactions may be enzymatic or nonenzymatic. Fresh smears must be used to detect enzymatic activity, whereas nonenzymatic procedures may be performed on specimens that have been stored at room temperature.
- MPO stains primary granules and is useful in differentiating granulocytic from lymphoid cells.
- SBB stains lipids and results parallel those with the MPO stain.
- Esterases help differentiate granulocytes and their precursors from cells of monocytic origin. Butyrate esterase testing gives positive results in monocytes but not in granulocyte precursors, whereas naphthol AS-D chloroacetate esterase stains granulocyte precursors.

Now that you have completed this chapter, go back and read again the case study at the beginning and respond to the questions presented.

REVIEW QUESTIONS

Answers can be found in the Appendix.

1. According to the WHO classification, except in leukemias with specific genetic anomalies, the minimal percentage of blasts necessary for a diagnosis of acute leukemia is:
 - a. 10%
 - b. 20%
 - c. 30%
 - d. 50%
2. A 20-year-old patient has an elevated WBC count with 70% blasts, 4% neutrophils, 5% lymphocytes, and 21% monocytes in the peripheral blood. Eosinophils with dysplastic changes are seen in the bone marrow. AML with which of the following karyotypes would be most likely to be seen?
 - a. AML with t(8;21)(q22;q22)
 - b. AML with t(16;16)(p13;q22)
 - c. AML with t(15;17)(q22;q12)
 - d. AML with t(9;11)(p22;q23)
3. Which of the following would be considered a sign of potentially favorable prognosis in children with ALL?
 - a. Hyperdiploidy
 - b. Presence of CD 19 and 20
 - c. Absence of trisomy 8
 - d. Presence of *BCR/ABL* gene
4. Signs and symptoms of cerebral infiltration with blasts are more commonly seen in:
 - a. AML with recurrent cytogenetic abnormalities
 - b. Therapy-related myeloid neoplasms
 - c. AML with myelodysplasia-related changes
 - d. ALL
5. An oncology patient exhibiting signs of renal failure with seizures after initial chemotherapy may potentially develop:
 - a. Hyperleukocytosis
 - b. Tumor lysis syndrome
 - c. Acute leukemia secondary to chemotherapy
 - d. Myelodysplasia
6. Disseminated intravascular coagulation is more often seen in association with leukemia characterized by which of the following mutations?
 - a. t(12;21)(p13;q22)
 - b. t(9;22)(q34;q11.2)
 - c. inv(16)(p13;q22)
 - d. t(15;17)(q22;q12)

7. Which of the following leukemias affects primarily children, is characterized by an increase in monoblasts and monocytes, and often is associated with gingival and skin involvement?
 - a. Pre-B lymphoblastic leukemia
 - b. Pure erythroid leukemia
 - c. AML with t(9;11)(p22;q23)
 - d. AML with t(15;17)(q22;q12)
8. A 20-year-old patient presents with fatigue, pallor, easy bruising, and swollen gums. Bone marrow examination reveals 82% cells with delicate chromatin and prominent nucleoli that are CD14⁺, CD4⁺, CD11b⁺, and CD36⁺. Which of the following acute leukemias is likely?
 - a. Minimally differentiated leukemia
 - b. Leukemia of ambiguous lineage
 - c. Acute monoblastic/monocytic leukemia
 - d. Acute megakaryoblastic leukemia
9. Pure erythroid leukemia is a disorder involving:
 - a. Pronormoblasts only
 - b. Pronormoblasts and basophilic normoblasts
 - c. All forms of developing RBC precursors
 - d. Equal numbers of pronormoblasts and myeloblasts
10. A patient with normal chromosomes has a WBC count of $3.0 \times 10^9/L$ and dysplasia in all cell lines. There are 60% blasts of varying sizes. The blasts stain positive for CD61. The most likely type of leukemia is:
 - a. Acute lymphoblastic
 - b. Acute megakaryoblastic
 - c. Acute monoblastic
 - d. AML with t(15;17)
11. SBB stains which of the following component of cells?
 - a. Glycogen
 - b. Lipids
 - c. Structural proteins
 - d. Enzymes
12. The cytochemical stain α -naphthyl butyrate is a nonspecific esterase stain that shows diffuse positivity in cells of which lineage?
 - a. Erythroid
 - b. Monocytic
 - c. Granulocytic
 - d. Lymphoid

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Mature Lymphoid Neoplasms

Magdalena Czader

OUTLINE

Morphologic and Immunophenotypic Features of Normal Lymph Nodes

Cortex

Paracortex

Medulla

Sinuses

Lymph Node Processing Reactive Lymphadenopathies

Follicular Pattern

Paracortical Pattern

Sinusoidal Pattern

Mixed Pattern

Lymphomas

Mature B Cell Lymphomas

Mature T Cell and Natural

Killer Cell Lymphomas

Hodgkin Lymphoma

OBJECTIVES

After completion of this chapter, the reader will be able to:

1. Describe normal lymph node morphology and discuss the function of various compartments and constituent cells.
2. Outline the most common histologic patterns of reactive lymphadenopathies.
3. Describe the peripheral blood findings in chronic lymphocytic leukemia and hairy cell leukemia.
4. Describe the approach to the diagnosis of lymphomas as outlined by the World Health Organization classification.
5. Discuss the most commonly occurring mature B and T cell neoplasms, including epidemiology, clinical presentation, pathophysiology, lymph node histologic features, peripheral blood or bone marrow findings, and diagnostic test results.
6. Interpret diagnostic test results to identify lymphoproliferative disorders.

CASE STUDY

After studying the material in this chapter, the reader should be able to respond to the following case study:

A 46-year-old previously healthy man came for evaluation of an enlarged left cervical lymph node. The patient had discovered this isolated lymphadenopathy 2 weeks previously and did not complain of any other symptoms. The lymph node measured approximately 2 cm. The findings of his physical examination were otherwise unremarkable. The lymph node was excised, and microscopic examination showed the histologic features presented in [Figure 36-1, A](#). Immunohistochemical stains showed CD20 ([Figure 36-1, B](#)), CD10, and BCL-6 positivity and focal CD30 antigen expression.

1. What is your diagnosis based on the histologic and immunophenotypic features?

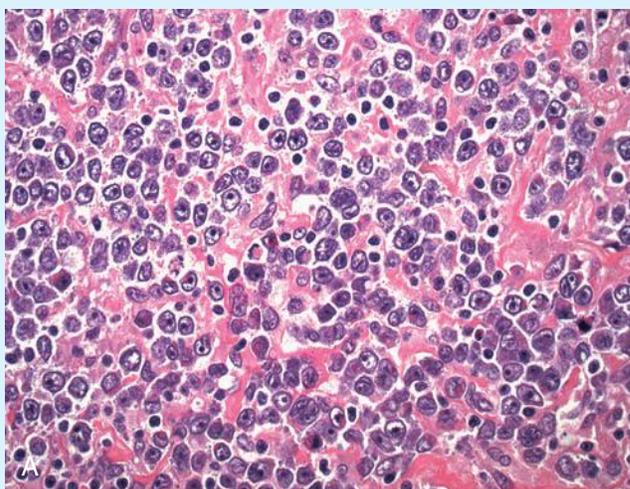


Figure 36-1 Histologic lymph node findings for the patient in the case study. **A**, Lymph node (hematoxylin and eosin stain, $\times 500$).

Continued

CASE STUDY—cont'd

After studying the material in this chapter, the reader should be able to respond to the following case study:

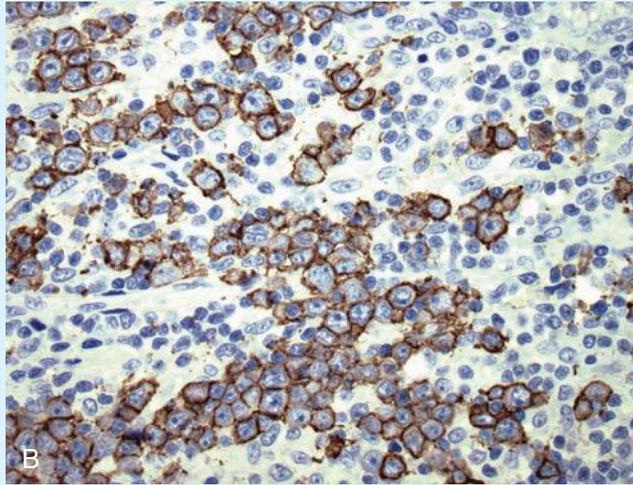


Figure 36-1, cont'd B, CD20 antigen expression in the lymphoid population (immunoperoxidase stain, $\times 500$).

2. What additional immunophenotypic features that confirm the diagnosis could be seen using flow cytometry?
3. Is it likely that this patient would show disseminated disease, including bone marrow involvement?

Lymphomas are neoplasms of the lymphoid system. Original microscopic observations and immunophenotypic and molecular studies confirmed that these malignancies recapitulate specific stages of normal lymphoid differentiation. The diagnosis is based on a combination of biologic features such as morphology, immunophenotype and molecular genetic characteristics, and clinical information.¹ Therefore, during initial sample processing, the appropriate steps must be taken to ensure tissue preservation and availability for microscopic examination and immunophenotypic and molecular studies.

Knowledge of normal lymphoid differentiation is a prerequisite for understanding the lymphoid neoplasms. This chapter describes the morphologic and immunophenotypic features of normal lymph nodes and selected common lymphomas and lymphoproliferative disorders. Reactive lymphoid hyperplasias, which can resemble lymphoid neoplasms, also are discussed.

MORPHOLOGIC AND IMMUNOPHENOTYPIC FEATURES OF NORMAL LYMPH NODES

Lymphoid organs serve as sites of antigen recognition, antigen processing, and lymphopoiesis. Most of the lymphoid tissue is concentrated in lymph nodes, which are round to oval encapsulated organs serving as primary sites of immunologic response. They are particularly prominent at sites with an environmental interface. Large groups of lymph nodes are found draining specific peripheral areas (e.g., cervical, axillary, or inguinal). Similarly, internal organs are served by regional lymph

nodes (e.g., mediastinal, hilar, and mesenteric). Respiratory and digestive tracts have additional aggregates of lymphoid tissue located directly in the mucosa called *mucosa-associated lymphoid tissue* (MALT). These aggregates are the primary sites of antigenic contact and drain directly into regional lymph nodes.

Histologic components of a lymph node include cortex, paracortex, medullary cords, and sinuses (Figure 36-2). They are both structural and functional compartments serving as sites of immunologic reactions for specific antigenic stimuli.

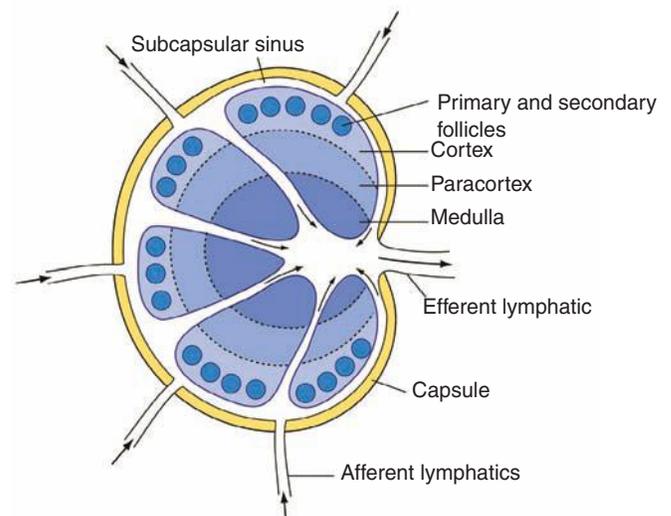


Figure 36-2 Diagram of a normal lymph node showing cortical, paracortical, and medullary compartments.

Cortex

The lymph node is surrounded by a capsule of fibrous tissue. Immediately below the capsule is the cortex, the most superficial portion of the lymph node consisting of primary and secondary follicles. Primary follicles are microscopic aggregates of small naive B lymphocytes. These lymphocytes express pan-B cell markers, including CD19 and CD20 and are frequently CD5⁺ (Figure 36-3). The formation of secondary follicles with germinal centers and their functions are assisted by follicular dendritic cell meshworks, best visualized by immunohistochemical stains such as CD21.^{2,3} On antigen encounter, naive B lymphocytes undergo transformation, proliferation, and differentiation into precursors of antibody-producing plasma cells and memory B cells (Figure 36-3). The remaining naive B cells are displaced into the periphery of the germinal center and form the mantle zone.

Germinal center B cells have a specific immunophenotype. In addition to pan-B cell markers, they express germinal center cell antigens CD10 and BCL6, and, in contrast to circulating B cells, they lack anti-apoptotic BCL2 protein. Functional compartments of the germinal center include the dark zone occupied by centroblasts, large B cells with round vesicular nuclei, small nucleoli adjacent to nuclear membrane, and basophilic cytoplasm (Figure 36-4). The dark zone is a site of high proliferative activity and somatic mutations of B cell immunoglobulin variable regions. The latter process allows for the production of immunoglobulins with the best affinity for a particular antigen.

After completing somatic mutations, centroblasts differentiate into centrocytes, smaller cells with dense chromatin and irregular nuclear outlines, which form the light zone (Figure 36-4). Subsequently, centrocytes with low-affinity (“unfit”) surface immunoglobulins undergo apoptosis and are phagocytized by germinal center macrophages (tingible-body macrophages). The presence of numerous macrophages with apoptotic debris contributes to

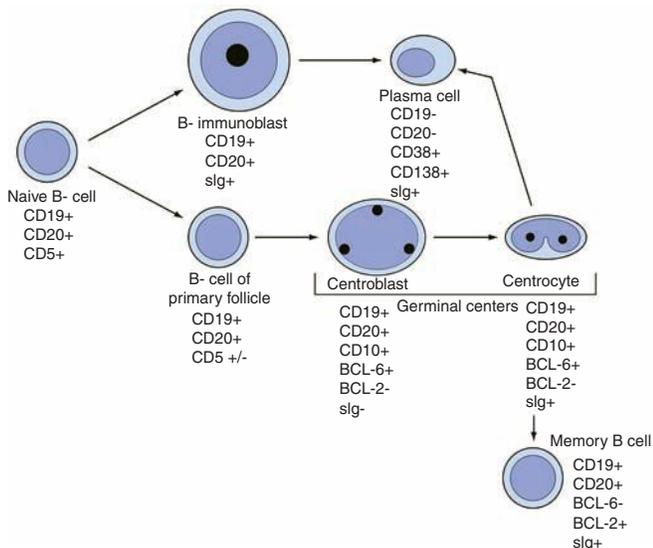


Figure 36-3 Differentiation stages of mature B cells. Note changes in immunophenotype at specific stages of differentiation. *BCL*, B cell lymphoma; *slg*, surface immunoglobulin.

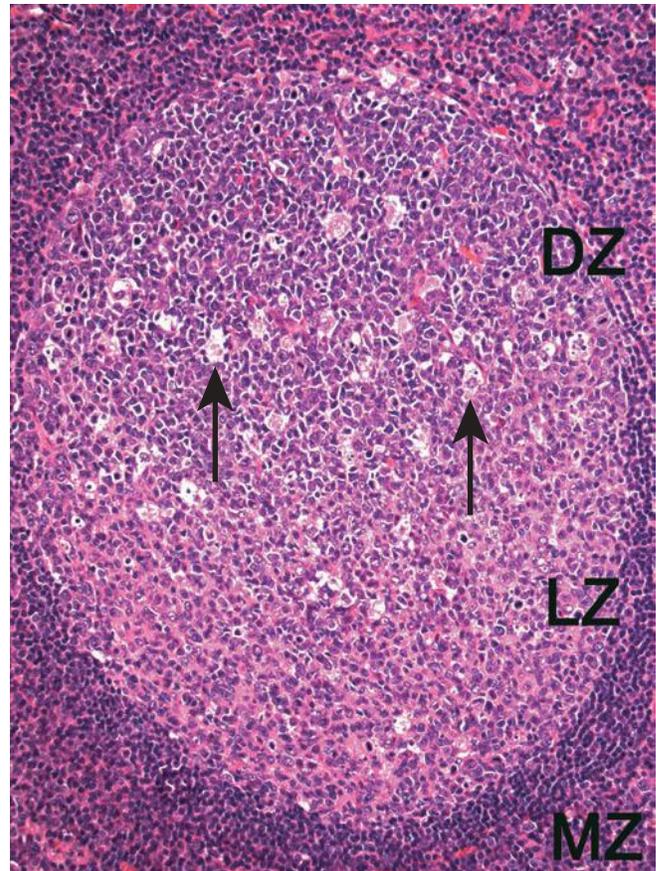


Figure 36-4 Secondary follicle with well-developed polarization in germinal center showing dark zone (DZ) and light zone (LZ). Note the presence of numerous tingible body macrophages (arrows). A distinct mantle zone (MZ) also is present at the periphery of the germinal center (hematoxylin and eosin stain, $\times 100$).

the characteristic “starry sky” pattern of the germinal center. Centrocytes with immunoglobulins with high affinity for a particular antigen lose their germinal center antigens (CD10 and BCL6) and differentiate into memory B cells that form a marginal zone at the periphery of the mantle zone. Marginal zone lymphocytes are medium sized with abundant clear cytoplasm and indented nuclei.

In the final step of B cell differentiation are plasma cells that reside in medullary cords of lymph nodes and that also migrate to bone marrow. Plasma cells are negative for pan-B cell antigens and surface immunoglobulins; however, they express CD138, CD38, and cytoplasmic immunoglobulins.

Paracortex

The paracortex occupies the area separating the follicles and extends toward medullary cords. This compartment generates immunocompetent T cells and is occupied predominantly by T cells, interdigitating dendritic cells (antigen-presenting cells), and high-endothelial venules. The last are specialized vessels serving as a gate of entry for lymphocytes from peripheral blood into lymph nodes. T cells express pan-T cell antigens such as CD3, CD5, CD2, and CD7. Both CD4⁺ and CD8⁺ T lymphocytes are seen in paracortex. Similar to B cells, T cells

transform in response to antigen stimulation. In this process, small lymphocytes become immunoblasts: large lymphoid cells with vesicular nuclei; prominent, often single, nucleoli; and abundant basophilic cytoplasm. The paracortex also contains numerous B immunoblasts.

Medulla

The medulla represents the innermost portion of the lymph node surrounding the hilum. This area is composed of medullary cords with plasma cells and medullary sinuses.

Sinuses

The filtration of lymphatic fluid through lymph nodes is accomplished via afferent lymphatics communicating with a subcapsular sinus, which is situated immediately beneath the capsule (Figure 36-2). The subcapsular sinus drains into cortical sinuses, which run through the cortex and empty to medullary sinuses. The latter converge into the efferent lymphatic vessel at the hilum. The sinuses are filled with macrophages or sinus histiocytes. These cells play an important role in antigen capture and processing.

LYMPH NODE PROCESSING

Current approach to diagnosis of lymphomas incorporates routine light microscopic examination and ancillary techniques. During processing of excised lymph nodes, appropriate steps should be taken to ensure adequate preservation of the tissue and its availability for all necessary studies. The appropriate transport conditions need to be maintained to preserve tissue integrity and prevent drying. Immediately after excision, the lymph node should be transported to the pathology laboratory in a sealed sterile jar on gauze pads moistened with sterile saline or in tissue culture media. The fresh lymph node is cut into 3-mm-thick sections for the evaluation of nodal architecture. If areas of granulomas or suppuration are present, a portion of the tissue should be sent for cultures.

Touch imprints can be prepared to ensure the adequacy of the specimen and to perform special studies. To obtain an adequate imprint, a freshly cut tissue surface is gently touched to the glass slide and pulled away. Touch imprints can be fixed in formalin or alcohol solution or air-dried for subsequent Wright-Giemsa staining. Storing of fixed touch imprints for immunocytochemical studies is optional because currently immunophenotyping is most commonly performed on paraffin-embedded tissue or using flow cytometry. The latter is particularly helpful in confirming monoclonal light chain expression.

Several thin lymph node sections are placed in 10% buffered formalin for paraffin embedding. Some pathology laboratories fix additional tissue samples in a variety of fixatives with protein-precipitating properties (B5 fixative, zinc chloride formalin) for better preservation of cytologic detail.⁴ Regardless of fixative used, thin sectioning of a fresh lymph node is crucial for proper tissue permeation and fixation. A portion of lymph node is placed in culture medium (Roswell Park Memorial Institute medium) and transported to a flow cytometry laboratory for immunophenotyping. The remaining fresh tissue can be stored at -70°C for further studies.

REACTIVE LYMPHADENOPATHIES

Lymphadenopathy, lymph node enlargement, can occur in benign/reactive and malignant conditions. Reactive lymphadenopathies can affect any compartment of a lymph node and present as expansion of normal nodal structures. Reactive hyperplasias are classified into several patterns, as follows:

1. Follicular
2. Paracortical
3. Sinusoidal
4. Mixed

Follicular Pattern

Follicular hyperplasia is the most common form of reactive lymphadenopathies. It is frequently seen in lymph nodes and tonsils of children and adolescents as a reaction to infections. In adults, it occurs in association with infections, autoimmune disorders (rheumatoid arthritis, systemic lupus erythematosus), syphilis, and early human immunodeficiency virus (HIV) infection. Microscopically, an expansion of reactive follicles can be prominent and extend beyond the cortex into the medulla (Figure 36-5). Secondary follicles retain all the hallmarks of reactive germinal centers, including distinct polarization, presence of tingible-body macrophages, abundant mitotic figures, and preserved mantle zones (Figure 36-4).

Paracortical Pattern

Paracortical expansion is associated with viral infections (e.g., infectious mononucleosis) and drug reactions and is also seen in patients with chronic skin diseases (dermatopathic lymphadenopathy). In addition to small lymphocytes, the paracortex shows numerous immunoblasts, increased mitotic activity, and vascular proliferation (Figure 36-6). Focal areas of necrosis may also be seen. In dermatopathic lymphadenopathy, the paracortex has a characteristic mottled appearance as a result of an increased number of large cells with abundant clear cytoplasm scattered among small lymphoid cells (Figure 36-7).

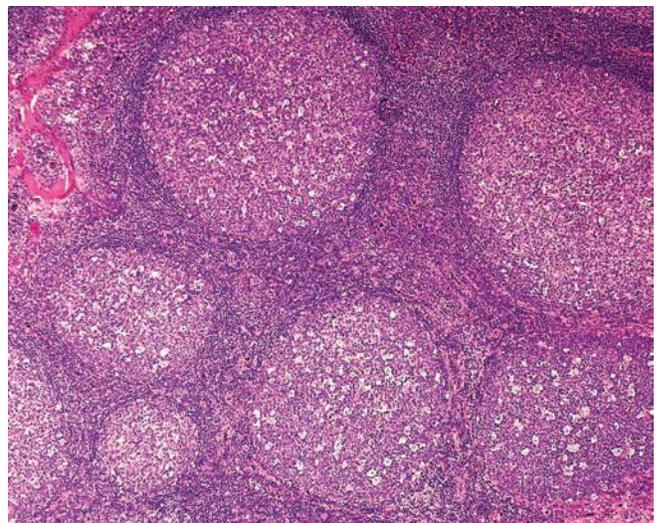


Figure 36-5 Reactive follicular hyperplasia with numerous secondary follicles scattered throughout the lymph node (hematoxylin and eosin stain, $\times 40$).

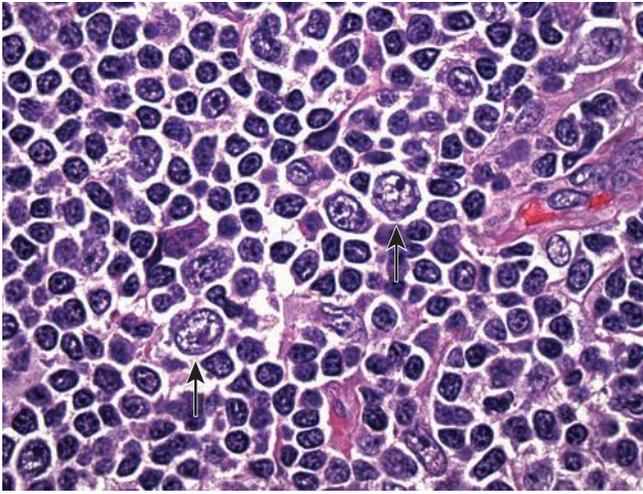


Figure 36-6 Immunoblasts (*arrows*) scattered in the paracortex (hematoxylin and eosin stain, $\times 1000$).

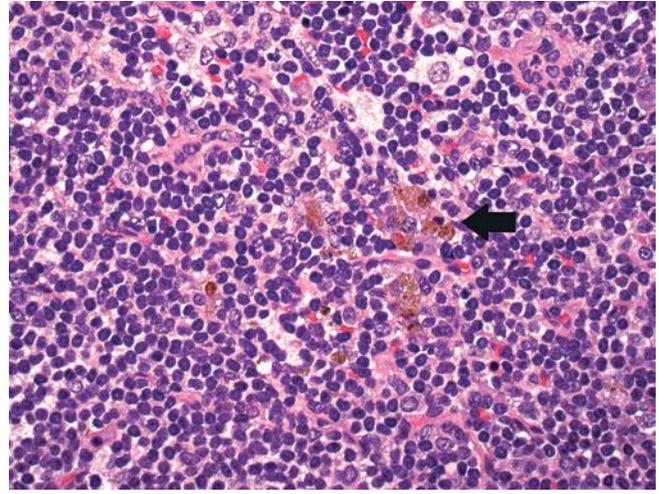


Figure 36-8 Dermatopathic lymphadenopathy in a patient with chronic skin rash. Scattered pigment-laden macrophages (*arrow*) are present in the paracortical area (hematoxylin and eosin stain, $\times 400$).

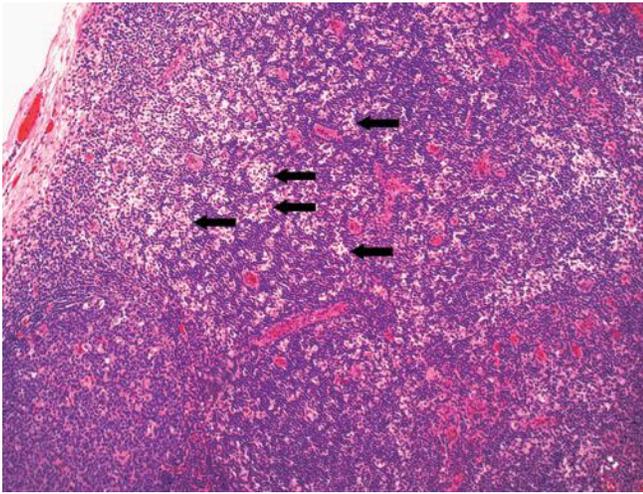


Figure 36-7 Paracortical hyperplasia. Note mottled appearance of the paracortex resulting from multiple scattered histiocytes with abundant cytoplasm (*arrows*) (hematoxylin and eosin stain, $\times 40$).

These cells include histiocytes, often carrying melanin pigment, and Langerhans cells (Figure 36-8). Scattered immunoblasts, plasma cells, eosinophils, and vascular proliferation are also encountered.

Sinusoidal Pattern

Expanded subcapsular, cortical, and medullary sinuses are often seen in lymph nodes draining limbs, abdominal organs, various inflammatory lesions, and malignancies. In select cases, the prominent sinuses compress the nodal parenchyma. They may be completely filled with histiocytes showing abundant cytoplasm, a small oval nucleus with inconspicuous nucleolus, and delicate chromatin. Monocytoid B cells with abundant cytoplasm and oval indented nuclei that may mimic histiocytes are seen in HIV-associated lymphadenopathy and *Toxoplasma* lymphadenitis (Figure 36-9, A). Numerous malignant lesions show predilection for sinuses, such as Langerhans cell histiocytosis, B and T cell lymphomas, and carcinomas;

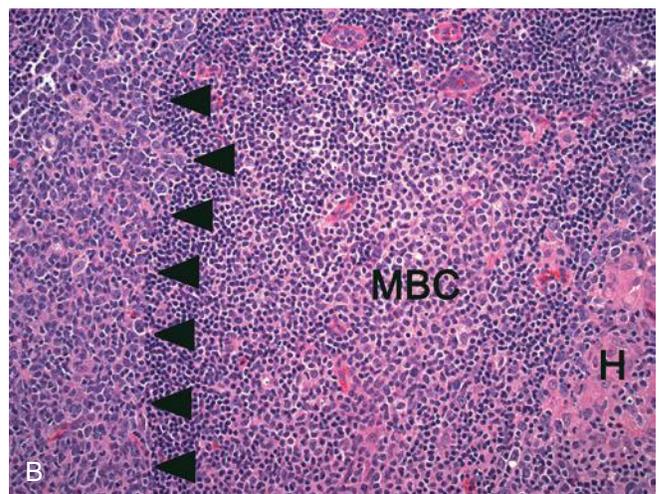
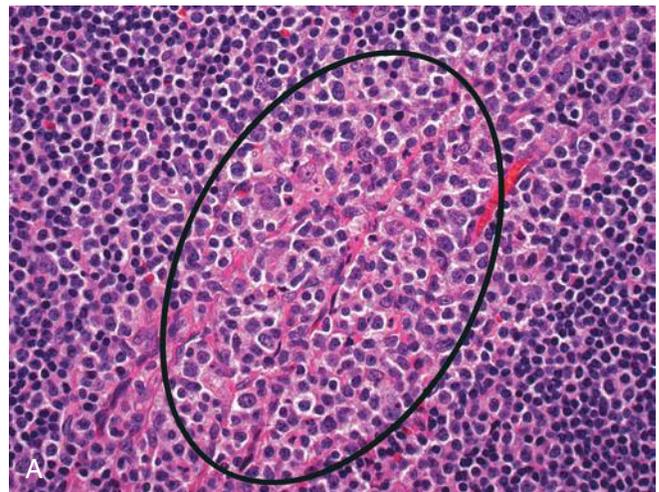


Figure 36-9 *Toxoplasma* lymphadenitis. **A**, Monocytoid B cells are medium sized with irregular nuclear outlines and abundant cytoplasm (shown in *center circle*) (hematoxylin and eosin stain, $\times 400$). **B**, Classic triad of reactive changes seen in *Toxoplasma* lymphadenitis includes follicular hyperplasia with focal aggregates of histiocytes (H) and monocytoid B cells (MBC). An irregular outline of a secondary follicle is seen on the left (*arrowheads*) (hematoxylin and eosin stain, $\times 100$).

therefore, a thorough evaluation of expanded sinuses under high magnification is always necessary.

Mixed Pattern

A classic example of mixed-pattern hyperplasia is seen in *Toxoplasma gondii* infection, a common protozoal infection typically seen after ingestion of raw meat or contamination by cat feces. The expansion of all lymph node compartments is seen (Figure 36-9). Florid follicular hyperplasia is accompanied by paracortical expansion, aggregates of histiocytes encroaching on germinal centers, and expanded sinuses. Sinuses are focally filled with a specific subset of B cells, so-called monocytoid B cells.

LYMPHOMAS

Approximately 86,000 new cases of lymphoma are diagnosed annually in the United States.⁵ Most lymphomas develop in previously healthy individuals. The strongest risk factor for development of lymphoproliferative disorder is altered immune function as seen in immunocompromised patients or individuals with autoimmune diseases.^{6,7} Similarly, certain viral and bacterial infections are associated with a higher risk for the

development of lymphoma.⁸ Accumulating evidence indicates that exposure to chemicals and herbicides may predispose to lymphoid neoplasms. Most lymphomas present in lymph nodes. Certain types show a predilection for extranodal sites. The frequency of bone marrow and peripheral blood (leukemic phase) involvement varies, depending on the lymphoma subtype.

Over the years, numerous classification systems have been proposed based mainly on the morphology and clinical characteristics (e.g., Rappaport classification, Kiel classification, Working Formulation). With increased understanding of the development and function of the immune system, however, it became clear that lymphomas, like myeloid neoplasms, recapitulate normal stages of lymphoid differentiation. In addition, the elucidation of specific molecular events occurring in lymphomagenesis helped in devising clinically relevant classification, especially for morphologically heterogeneous entities. Currently, numerous types of lymphoma are distinguished based on morphology, immunophenotype, molecular genetics, and clinical and laboratory characteristics. The integration of these features is mandatory for comprehensive lymphoma diagnosis. On the basis of cellular origin, lymphomas can be categorized into lesions of lymphoid precursors and neoplasms of mature lymphoid cells (Table 36-1).

TABLE 36-1 2008 World Health Organization Classification of Mature Lymphoid Neoplasms

Type of Lymphoma	Examples
Mature B cell lymphomas	Chronic lymphocytic leukemia/small lymphocytic lymphoma B cell prolymphocytic leukemia Splenic B cell marginal zone lymphoma Hairy cell leukemia Splenic B cell lymphoma/leukemia, unclassifiable Splenic diffuse red pulp small B cell lymphoma Hairy cell leukemia-variant Lymphoplasmacytic lymphoma Heavy chain diseases Gamma heavy chain disease Mu heavy chain disease Alpha heavy chain disease Plasma cell neoplasms Monoclonal gammopathy of undetermined significance (MGUS) Plasma cell myeloma Solitary plasmacytoma of bone Extraosseous plasmacytoma Monoclonal immunoglobulin deposition diseases Extranodal marginal zone lymphoma of mucosa-associated lymphoid tissue (MALT lymphoma) Nodal marginal zone lymphoma Follicular lymphoma Primary cutaneous follicle center lymphoma Mantle cell lymphoma Diffuse large B cell lymphoma (DLBCL), not otherwise specified T cell/histiocyte-rich large B cell lymphoma Primary DLBCL of the central nervous system Primary cutaneous DLBCL, leg type Epstein-Barr virus (EBV)-positive DLBCL of the elderly DLBCL associated with chronic inflammation Lymphomatoid granulomatosis Primary mediastinal (thymic) large B cell lymphoma

TABLE 36-1 2008 World Health Organization Classification of Mature Lymphoid Neoplasms—cont'd

Type of Lymphoma	Examples
	Intravascular large B cell lymphoma
	ALK-positive large B cell lymphoma
	Plasmablastic lymphoma
	Large B cell lymphoma arising in human herpesvirus 8–associated multicentric Castleman disease
	Primary effusion lymphoma
	Burkitt lymphoma
	B cell lymphoma, unclassifiable, with features intermediate between those of DLBCL and Burkitt lymphoma
	B cell lymphoma, unclassifiable, with features intermediate between those of DLBCL and classical Hodgkin lymphoma
Mature T cell lymphomas	T cell prolymphocytic leukemia
	T cell large granular lymphocytic leukemia
	Chronic lymphoproliferative disorder of natural killer (NK) cells
	Aggressive NK cell leukemia
	EBV-positive T cell lymphoproliferative diseases of childhood
	Systemic EBV-positive T cell lymphoproliferative diseases of childhood
	Hydroa vacciniforme–like lymphoma
	Adult T cell leukemia/lymphoma
	Extranodal NK/T cell lymphoma, nasal type
	Enteropathy-associated T cell lymphoma
	Hepatosplenic T cell lymphoma
	Subcutaneous panniculitis-like T cell lymphoma
	Mycosis fungoides
	Sézary syndrome
	Primary cutaneous CD30 ⁺ T cell lymphoproliferative disorders
	Primary cutaneous peripheral T cell lymphomas, rare subtypes
	Primary cutaneous gamma-delta T cell lymphoma
	Primary cutaneous CD8 ⁺ aggressive epidermotropic cytotoxic T cell lymphoma
	Primary cutaneous CD4 ⁺ small/medium T cell lymphoma
	Peripheral T cell lymphoma, not otherwise specified
	Angioimmunoblastic T cell lymphoma
	Anaplastic large cell lymphoma, ALK positive
	Anaplastic large cell lymphoma, ALK negative

In this chapter, only mature B cell and T cell neoplasms are discussed; the precursor malignancies are covered in Chapter 35.

Mature B Cell Lymphomas

Mature B cell lymphomas are derived from various stages of B cell differentiation. Although they show significant morphologic and immunophenotypic heterogeneity, all B cell lymphomas produce monoclonal light chain immunoglobulins, clonal immunoglobulin gene rearrangements, or both. Follicular lymphoma and diffuse large B cell lymphoma (DLBCL) are the most common subtypes of B cell lymphoma.⁹ Most cases are lymph node based and occur in elderly individuals. However, leukemic involvement (peripheral blood and bone marrow) can occur with any lymphoma type. The most common mature B cell neoplasms are discussed in the following paragraphs and are summarized in Table 36-2.

Chronic Lymphocytic Leukemia/Small Lymphocytic Lymphoma

Definition. Chronic lymphocytic leukemia (CLL) and small lymphocytic lymphoma (SLL) are characterized by accumulation

of small lymphoid cells in peripheral blood, bone marrow, and lymphoid organs. The exact cell of origin in CLL/SLL is not known, however gene expression profiling has shown that antigen-experienced B cells, both memory B cells and marginal zone B cells, are likely candidates.¹⁰ The World Health Organization (WHO) classification scheme considers CLL and SLL as one entity with different clinical presentations.¹ The diagnosis of CLL/SLL is based on the predominant site of involvement. CLL presents mostly in peripheral blood and bone marrow. SLL primarily involves lymph nodes and other lymphoid organs.

Morphology. In CLL, bone marrow and peripheral blood films show small lymphoid cells with a characteristically coarse chromatin (“soccer-ball” pattern), absent or inconspicuous nucleoli, and scant cytoplasm^{1,11} (Figure 36-10). According to the 2008 International Workshop on Chronic Lymphocytic Leukemia, up to 55% of the cells in CLL may include prolymphocytes (larger lymphoid cells with pale blue cytoplasm, less condensed chromatin, and a distinct nucleolus), lymphoid cells with cleaved nuclei, or large lymphoid cells with an

TABLE 36-2 Morphologic and Immunophenotypic Features of Mature B-Cell Lymphomas

Subtype	Architectural Features	Cytologic Characteristics	Immunophenotype/ Cytogenetics	Cell of Origin
Chronic lymphocytic leukemia/small lymphocytic lymphoma	Diffuse lymphocytic proliferation with growth centers	Small lymphoid cells	CD20 ⁺ , CD19 ⁺ , CD5 ⁺ , CD23 ⁺ , LEF1	Memory and marginal zone B cells
B cell prolymphocytic leukemia	Diffuse proliferation	Medium-sized lymphoid cells with distinct “punched-out” nucleoli and abundant cytoplasm	CD20 ⁺ , CD19 ⁺ , FMC7 ⁺ , CD5 ^{+/-}	Unknown mature B cell
Mantle cell lymphoma	Diffuse, nodular, or mantle zone pattern	Medium-sized lymphocytes with irregular nuclei	CD20 ⁺ , CD19 ⁺ , CD5 ⁺ , FMC7 ⁺ , SOX11, cyclin D1 ⁺ , t(11;14)	Mantle zone cell
Follicular lymphoma	Follicular pattern	Medium-sized lymphocytes with indented nuclei and variable numbers of large lymphoid cells	CD20 ⁺ , CD19 ⁺ , CD10 ⁺ , BCL6 ⁺ , BCL2 ⁺ , t(14;18)	Germinal center cell
Extranodal marginal zone lymphoma of mucosa-associated lymphoid tissue	Diffuse lymphoid proliferation, occasionally marginal zone or nodular pattern	Medium-sized lymphocytes with irregular nuclei and clear abundant cytoplasm	CD20 ⁺ , CD19 ⁺ , CD43 ^{+/-}	Marginal zone cell
Plasma cell myeloma, plasmacytoma	Sheets or large aggregates of plasma cells	Plasma cells, frequently with cytologic atypia	CD20 ⁻ , CD19 ^{+/-} , CD38 ⁺ , CD138 ⁺ , cytoplasmic light chain ⁺	Plasma cell
Diffuse large B cell lymphoma	Diffuse proliferation	Large lymphoid cells	CD20 ⁺ , CD19 ⁺ , CD10 ^{+/-} , BCL6 ^{+/-} , BCL2 ^{+/-} , CD5 ^{+/-}	Different stages of mature B cells
Burkitt lymphoma	Diffuse lymphoid proliferation with “starry sky” pattern	Medium-sized lymphocytes with evenly distributed chromatin, inconspicuous nucleoli	CD20 ⁺ , CD19 ⁺ , CD10 ⁺ , BCL6 ⁺ , BCL2 ⁻ , high proliferative activity, t(8:14)	Germinal center cell

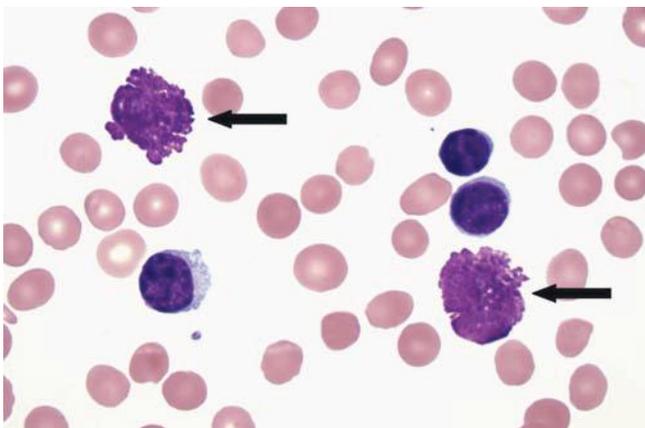


Figure 36-10 Chronic lymphocytic leukemia/small lymphocytic lymphoma. Peripheral blood film showing small lymphocytes and smudge cells (arrows) (Wright-Giemsa stain, $\times 1000$).

atypical appearance.¹² The French-American-British (FAB) Cooperative Group has proposed, and other groups have supported, a morphologic classification of CLL consisting of two major types: typical CLL (>90% small mature lymphocytes and <10% prolymphocytes) and an atypical CLL category.^{13,14,15,16} The 2008 WHO classification of lymphoid malignancies does not divide CLL into typical and atypical types.¹

Smudge cells, representing disintegrated lymphoid cells, are typically seen on peripheral blood films of CLL patients. Smudge cells are helpful in the diagnosis because they are not often seen in other subtypes of malignant lymphoma. The bone marrow biopsy specimen shows nodular, diffuse, or interstitial infiltrates of small lymphoid cells (Figure 36-11).

Lymph nodes involved by SLL show an effacement of normal nodal architecture by a diffuse proliferation of small, round lymphoid cells with coarse chromatin, indistinct nucleoli, and scant cytoplasm (Figure 36-12, A). In addition, scattered nodules (so-called pseudofollicles, growth centers, or proliferation centers) composed of medium-sized and large lymphoid cells with dispersed chromatin and distinct nucleoli are observed (Figure 36-12, B). The diffuse proliferation of small lymphoid cells with pseudofollicles is pathognomonic for SLL.

Diagnosis and Immunophenotype. CLL is diagnosed based on a sustained increase in the monoclonal B lymphocytes with CLL immunophenotype which is equal or greater than 5000/uL. The CLL immunophenotype includes an expression of CD19, CD20, and CD23, with aberrant expression of CD5.¹ Expression of CD20 and CD79b is weaker than in normal B cells.¹ Immunophenotyping also demonstrates

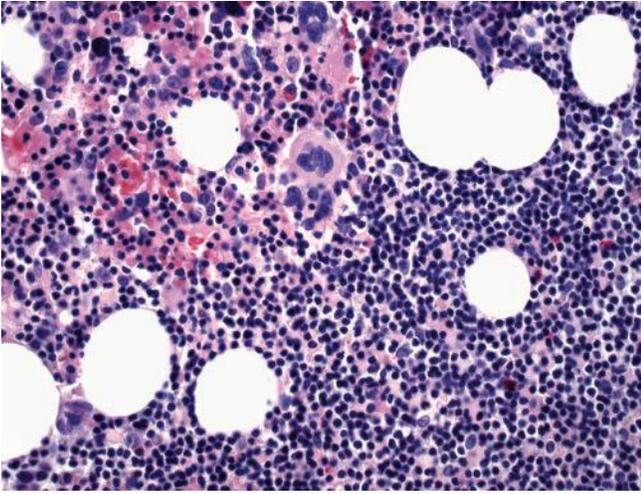


Figure 36-11 Chronic lymphocytic leukemia/small lymphocytic lymphoma. Bone marrow biopsy specimen with nodular (*right*) and interstitial lymphoid infiltrate composed of small lymphocytes (hematoxylin and eosin stain, $\times 400$).

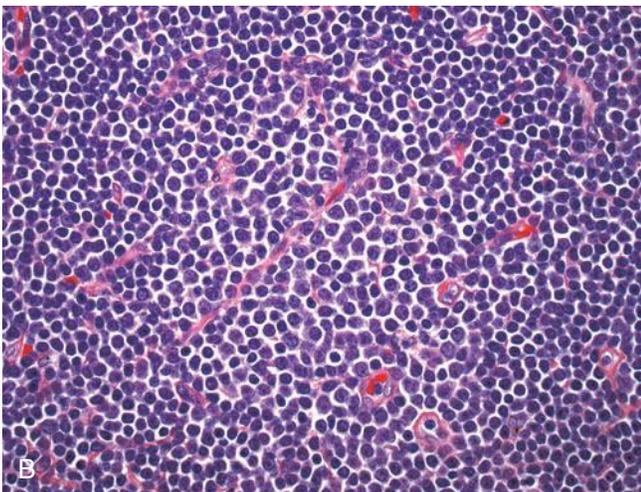
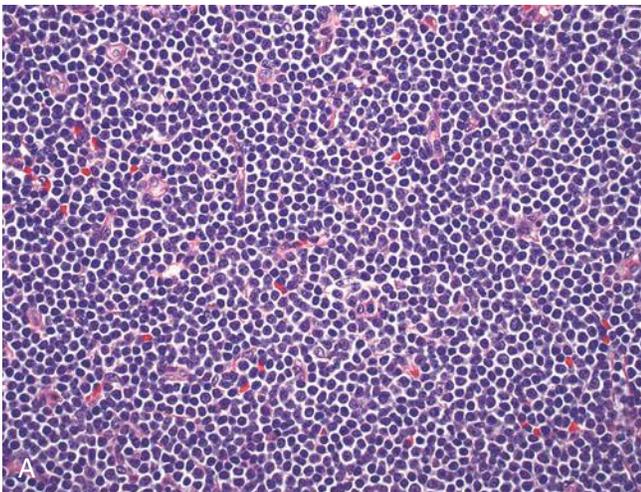


Figure 36-12 Chronic lymphocytic leukemia/small lymphocytic lymphoma in a lymph node. **A**, Diffuse proliferation of small lymphoid cells (hematoxylin and eosin stain, $\times 100$). **B**, Proliferation center with large and medium-sized lymphoid cells in a background of small lymphocytes (hematoxylin and eosin stain, $\times 400$).

expression of kappa or lambda light chains (clonal light chain restriction).^{1,12}

CD23 and LEF1 (lymphoid-enhancer-binding factor 1) expression and the absence of FMC7, cyclin D1, and SOX11 distinguish CLL/SLL from mantle cell lymphoma. The presence of less than 5000/ μL of circulating monoclonal B cells with a CLL/SLL immunophenotype can be found in a small proportion of healthy individuals and has been designated a monoclonal B-cell lymphocytosis.^{1,17} Therefore, the demonstration of these cells by flow cytometry must always be interpreted in the context of other clinical and laboratory features.¹⁷

Clinical Features and Prognosis. CLL/SLL generally affects older adults, however approximately 24% of patients are less than 55 years old.¹⁸ In CLL most patients are asymptomatic at diagnosis. The first indication of disease is often an incidental finding of lymphocytosis on a routine CBC in a blood count ordered for a different reason.

CLL is a heterogeneous disease in terms of clinical behavior. A variety of features have been used over the years to predict patient outcomes, including staging systems based on the extent of lymphoid organ involvement and degree of cytopenias. More recently, a growing number of biologic and molecular markers are being used which have enabled a more accurate assessment of disease status and prognosis.

The somatic mutation status of the variable region of the immunoglobulin heavy chain (*IGVH*) locus has divided the disease into two groups with considerably different outcomes.¹¹ Approximately 55% of CLL patients have mutated *IGVH*, indolent disease, and a median survival time of 24 years.^{19,20} On the other hand, patients with unmutated *IGVH* have aggressive disease and a median survival of approximately 8 years.^{19,20} At diagnosis, patients can be routinely screened using techniques such as fluorescent in situ hybridization (FISH) for the presence of chromosomal abnormalities, del13q14.3, del11q22-23, trisomy 12, and del17p13, which provide information regarding predicted survival (good, poor, intermediate, and very poor, respectively).²⁰ Next generation sequencing has recently identified additional significant genetic mutations such as *EZH2*, *NOTCH1*, *SF3B1*, and *BIRC3*, which can be used for an assessment of prognosis.²¹ The timing as well as selection of the most appropriate treatment approach has also been the subject of intense research. Independent of the course of the original disease, approximately 5% of patients with CLL/SLL develop a high-grade diffuse large B cell lymphoma (called *Richter syndrome*) with a survival of less than 1 year.¹⁶

Prolymphocytic Leukemia

Definition. Prolymphocytic leukemia (PLL) is a rare mature lymphoid leukemia that can be derived from B or T cells. Both B cell and T cell types involve peripheral blood, bone marrow, and spleen. Lymph node involvement is more commonly seen in T cell PLL. This lymphoproliferative disease is distinct from CLL, and its diagnosis requires that more than 55% of circulating lymphoid cells have the morphology of a prolymphocyte.

Morphology and Immunophenotype. The pathognomonic cell of B cell PLL is a prolymphocyte of medium size with round nucleus, moderately abundant cytoplasm, and distinct “punched-out” nucleolus (Figure 36-13). The cell size (twice that of a normal lymphocyte) and prominent central nucleolus allow PLL to be distinguished from CLL/SLL. The peripheral blood involvement in PLL is prominent, with a white blood cell count frequently in excess of $100 \times 10^9/L$. Bone marrow shows interstitial and/or nodular proliferation of prolymphocytes. Both white and red splenic pulp are infiltrated by PLL. B cell PLL is positive for pan-B cell markers CD20, CD19, CD22, and FMC7. The density of CD20 and surface light chain is higher than in typical cases of CLL/SLL. A proportion of cases are positive for CD5 antigen. In such cases, distinguishing the PLL from mantle cell lymphoma presenting with a leukemic involvement might be challenging and requires cytogenetic or molecular studies to exclude the presence of t(11;14) (refer to the section on mantle cell lymphoma).

Morphologic features of T cell PLL are not as distinct as those of the B cell type. Neoplastic cells seen in peripheral blood films are small to medium size, with round or irregular nuclei, the latter resembling Sézary cells. Prominent nucleoli are seen only in a proportion of cases. Cytoplasmic blebbing is common. Bone marrow, spleen, lymph node, and occasionally skin involvement is diffuse, with accentuation in nodal paracortical areas and around the vessels in dermis. The diagnosis of T cell PLL is challenging when attempted using morphologic features alone. A combination of morphologic, clinical, and immunophenotypic features is most helpful in making the diagnosis. T prolymphocytes are positive for T cell markers such as CD3, CD2, and CD5. In contrast to many T cell lymphomas, T cell PLL is positive for CD7 antigen. Most commonly CD4 antigen is expressed. A minority of cases can be double positive for CD4 and CD8, or positive for CD8.

Clinical Features and Prognosis. Like CLL/SLL, PLL is a disease of the elderly (mean age of presentation is 70 years). Overall prognosis is poor (median survival is 3 years for B cell

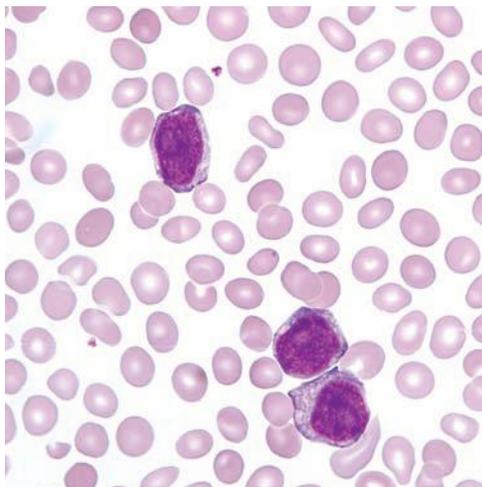


Figure 36-13 Peripheral blood film showing a characteristic morphology of prolymphocytes (medium size, abundant cytoplasm, distinct nucleoli) ($\times 1000$).

PLL), partly due to the high incidence of mutation in the tumor suppressor gene *TP53* and the unavailability of targeted therapy.²² T cell PLL is an aggressive disease with a median survival of 1 year when conventional chemotherapies are used. Recently, addition of monoclonal antibody therapy against CD52 (alemtuzumab) has significantly improved treatment response and survival of patients with T cell PLL.

Hairy Cell Leukemia

Definition. Hairy cell leukemia is characterized by small B lymphocytes with abundant cytoplasm and fine (“hairy”) cytoplasmic projections. The postulated cell of origin is the peripheral B cell of post-germinal center stage (memory B cell).

Morphology and Immunophenotype. Hairy cell leukemia cells are found predominantly in bone marrow and the red pulp of the spleen. A low number of neoplastic B cells are seen in peripheral blood. Lymph node involvement is rare. The bone marrow infiltrates are interstitial and are composed of small to medium-sized lymphoid cells with abundant cytoplasm (Figure 36-14, A). The bone marrow involvement may be subtle with preservation of normal hematopoiesis. As a result of the production of fibrogenic cytokines by leukemic cells, a bone marrow biopsy specimen shows an increase in reticulin fibers. The characteristic cytologic features of neoplastic cells are best appreciated in bone marrow aspirate and peripheral blood films. Neoplastic cells display an oval or indented nucleus, abundant cytoplasm, and fine, hairlike cytoplasmic projections (Figure 36-14, B).

Typical cases of hairy cell leukemia show strong positivity for B cell markers (CD19, CD20, CD22) coupled with bright expression of CD11c, CD25, CD103, tartrate-resistant acid phosphatase (TRAP, demonstrated by immunohistochemical analysis or cytochemical stain), DBA-44, CD123, and annexin A1. CD123 and annexin A1 are the most specific markers for classic hairy cell leukemia and can help in differentiating hairy cell leukemia from splenic marginal zone lymphoma. These antibodies, available for both flow cytometry and paraffin-embedded tissues, replaced the TRAP cytochemical stain previously commonly used to establish the diagnosis of hairy cell leukemia.

Clinical Features and Prognosis. Hairy cell leukemia is a rare lymphoproliferative disorder occurring in middle-aged individuals (median age, 55 years). The presenting signs include splenomegaly and pancytopenia. Durable remissions can be achieved using purine analogues. Conventional lymphoma therapy is not effective.

Mantle Cell Lymphoma

Definition. Mantle cell lymphoma is a lymphoproliferative disorder characterized by medium-sized lymphoid cells with irregular nuclear outlines derived from the follicular mantle zone.²³

Morphology and Immunophenotype. The main sites of presentation are lymph nodes. Bone marrow, peripheral blood, spleen, and gastrointestinal tract also are frequently involved. Most commonly, lymph nodes show a replacement of

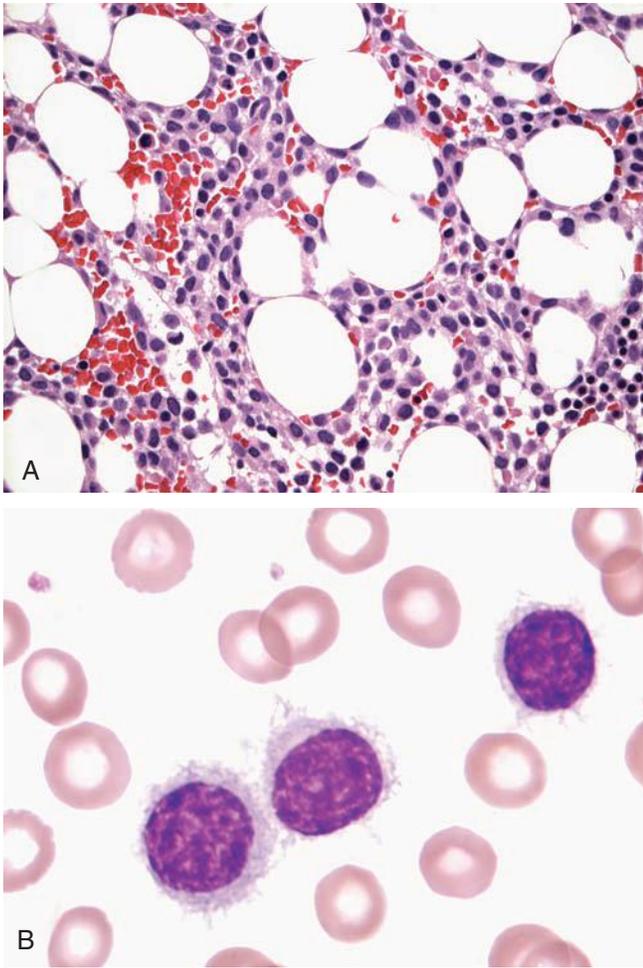


Figure 36-14 Hairy cell leukemia. **A**, Interstitial bone marrow infiltrate composed of widely spaced lymphoid cells with abundant cytoplasm and irregular nuclei (hematoxylin and eosin stain, $\times 1000$). **B**, Lymphoid cells of hairy cell leukemia show characteristic cytoplasmic projections, which are supported by the peripheral cytoplasmic network of polymerized actin (peripheral blood, Wright-Giemsa stain, $\times 1000$).

normal nodal architecture with a diffuse proliferation of monotonous, medium-sized lymphoid cells with irregular nuclear outlines (Figure 36-15, A). Occasionally, lymph nodes demonstrate a vaguely nodular pattern or partial preservation of nodal architecture with a prominent thickening of mantle zones (Figure 36-15, B). Peripheral blood involvement by mantle cell lymphoma can mimic PLL (Figure 36-15, C).

Like other B cell lymphoproliferative disorders, mantle cell lymphoma shows expression of pan-B cell markers (CD19, CD20) and high-density clonal surface light chains. There is coexpression of CD5 antigen; however, CD23 antigen is absent. In contrast to CLL/SLL, the expression of CD20 and light chains is strong, and there is immunoreactivity for cyclin D1 and SOX11. *Cyclin D1 (BCL1)* is a proto-oncogene involved in the regulation of G₁ to S phase transition. In mantle cell lymphoma, this gene is constitutively expressed through its translocation to the immunoglobulin heavy chain gene, t(11;14). This cytogenetic abnormality is present in the majority of mantle cell lymphomas. Cyclin D1-negative cases are positive for SOX11, a recently described diagnostic

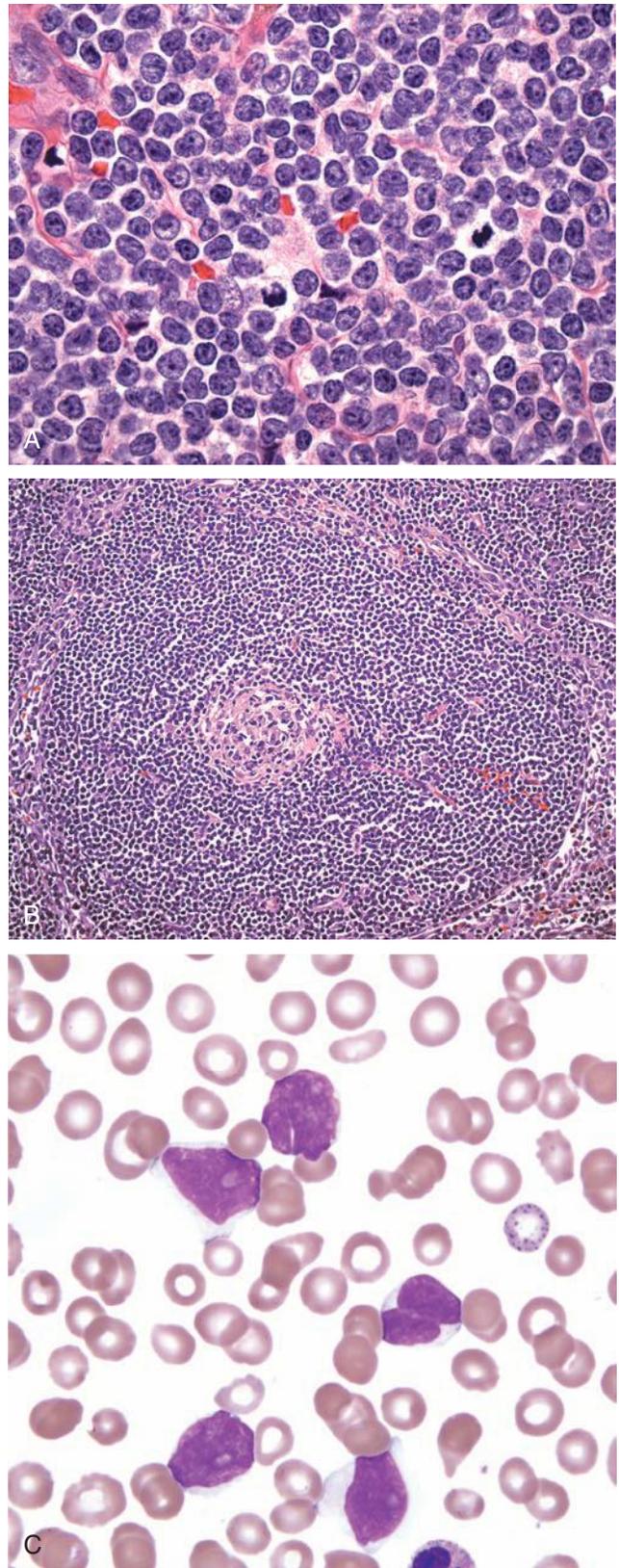


Figure 36-15 Mantle cell lymphoma. **A**, Diffuse proliferation of medium-sized lymphoid cells with irregular nuclei (hematoxylin and eosin stain, $\times 1000$). **B**, Mantle zone pattern. Regressed germinal center is seen in the center (hematoxylin and eosin stain, $\times 100$). **C**, Circulating mantle cell lymphoma cells with a few showing indented nuclei reminiscent of the cleaved nuclei seen in histologic sections (peripheral blood film, Wright-Giemsa stain, $\times 1000$).

and prognostic marker present in the majority of cases of mantle cell lymphoma.

Clinical Features and Prognosis. Mantle cell lymphoma is an aggressive lymphoproliferative disorder with a median survival time of 3 to 5 years. Most patients present with disseminated disease, including bone marrow involvement. This lymphoma is incurable with currently available chemotherapy, but stem cell transplantation is successful in a proportion of patients.

Follicular Lymphoma

Definition. Follicular lymphoma originates from germinal center B cells and in most cases recapitulates follicular architecture.

Morphology and Immunophenotype. Numerous closely spaced follicles replace the normal nodal architecture (Figure 36-16). The neoplastic proliferation may extend into perinodal adipose tissue. In contrast to the reactive secondary follicles, in follicular lymphoma the mantle zone and polarization are absent. The neoplastic follicles are composed of medium-sized lymphoid cells with angular or indented nuclei, cytologically similar to centrocytes, with a variable admixture of large lymphoid cells. The latter resemble centroblasts and show oval nuclei with vesicular chromatin and several nucleoli located close to nuclear membrane. The relative proportion of medium-sized and large lymphoid cells is of prognostic significance. Cases with high numbers of large cells show a more aggressive clinical course, similar to that of DLBCL. Therefore, follicular lymphomas are graded by counting the average number of large cells per high-power field.¹ Two grades are recognized: grade 1-2 shows rare scattered large lymphocytes, and grade 3 follicular lymphomas are composed of numerous centroblasts.

The immunophenotype reflects the follicle center cell origin of this disease. Pan-B cell markers (CD19, CD20) are present,

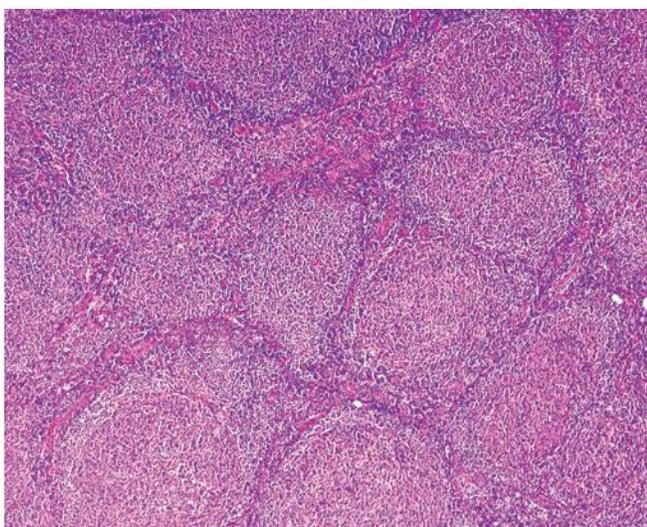


Figure 36-16 Follicular lymphoma. Nodular proliferation replacing normal elements of lymph node architecture (hematoxylin and eosin stain, $\times 40$).

along with the coexpression of CD10, BCL6, and clonal surface immunoglobulin. In contrast to reactive follicles, neoplastic cells express BCL2 protein. This protein is responsible for the decreased sensitivity of lymphoma cells to apoptosis and allows the accumulation of neoplastic lymphocytes. The expression of BCL-2 by follicular lymphoma cells is due to the t(14;18)(q32;q21), which places the *BCL2* gene under a promoter of the immunoglobulin heavy chain gene. This cytogenetic abnormality is present in 95% of cases.²⁴

Clinical Features and Prognosis. The median age at diagnosis is 59 years. Most patients present with disseminated disease. Bone marrow involvement is present in approximately 50% of cases. The course of the disease is indolent in grade 1-2 follicular lymphoma, whereas grade 3 cases are more aggressive and are treated in a manner similar to DLBCL with doxorubicin (Adriamycin)-based regimens.

Extranodal Marginal Zone Lymphoma of Mucosa-Associated Lymphoid Tissue

Definition. Three subtypes of marginal zone lymphomas are recognized: nodal, extranodal (MALT lymphoma), and splenic. In this chapter, we focus on the extranodal variant derived from marginal zone cells of MALT. In this lymphoma, the neoplastic proliferation is usually heterogeneous, encompassing small and medium-sized lymphocytes, plasma cells, and scattered large lymphoid cells. MALT lymphoma is frequently associated with autoimmune conditions (e.g., Sjögren syndrome, Hashimoto thyroiditis) or infections (*Helicobacter pylori* gastritis or hepatitis C).²⁵ Persistent immune stimulation leads to the accumulation of reactive lymphoid tissue and subsequently to the development of marginal zone lymphoma. The importance of continuous antigenic stimulation in the early stages of these lymphomas is shown by the remission of the disease, when associated infection is eradicated with antibiotic therapy.²⁶

Morphology and Immunophenotype. In most cases, the neoplastic population is composed of a mixture of medium-sized lymphocytes, plasma cells, and occasional large lymphoid cells. There is a predominance of medium-sized marginal zone cells with irregular nuclei (Figure 36-17, A). Residual reactive germinal centers may be present, which are colonized to variable degrees by neoplastic cells. A characteristic feature of MALT lymphoma is the presence of so-called lympho-epithelial lesions, representing the invasion of the neoplastic lymphocytes into the glandular epithelium (Figure 36-17, B). This feature is usually absent from reactive lymphoid proliferations associated with autoimmune processes or infections.

The neoplastic cells of marginal zone lymphoma express CD20, CD19, and monoclonal immunoglobulin chains. CD5 and CD10 are absent. CD43 antigen is coexpressed in 30% of cases and can be a helpful feature in diagnosing marginal zone lymphoma when there is a significant residual reactive component. In select cases, the demonstration of clonality by flow cytometry or by polymerase chain reaction (PCR) analysis of immunoglobulin heavy chain (IgH) gene rearrangements may be necessary to confirm the diagnosis.

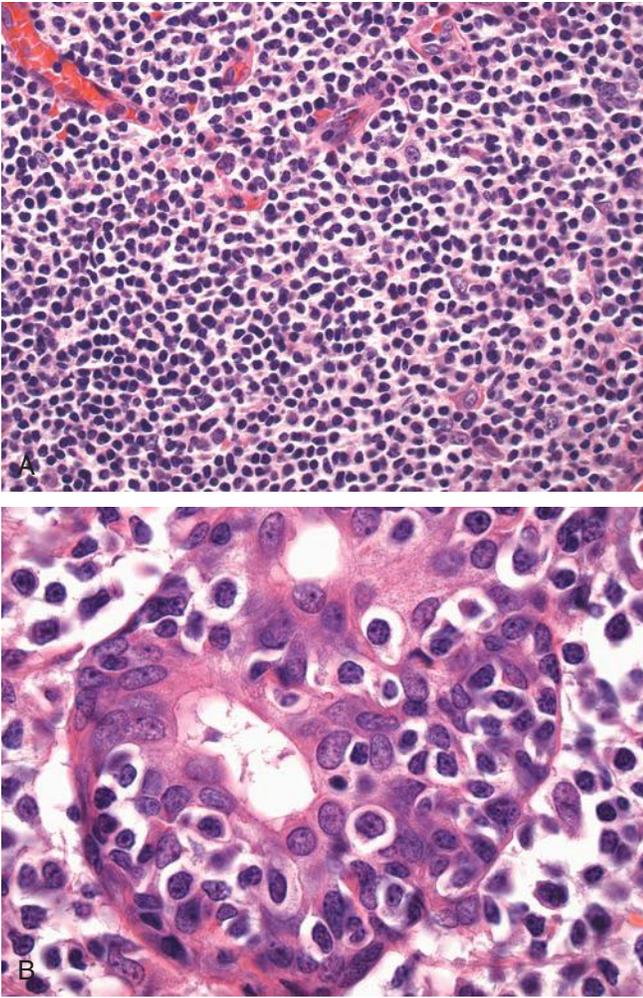


Figure 36-17 Mucosa-associated lymphoid tissue lymphoma. **A**, Heterogeneous population of medium-sized lymphoid cells with abundant clear cytoplasm and occasional large cells (hematoxylin and eosin stain, $\times 500$). **B**, Lymphoepithelial lesion. Malignant lymphocytes invading glandular epithelium (hematoxylin and eosin stain, $\times 1000$).

Antigenic stimulation and interference with the apoptotic pathway play an important role in the pathogenesis of MALT lymphoma. Approximately 30% of cases show a translocation involving apoptosis-inhibitor gene *API2* and the *MLT* gene, the $t(11;18)(q21;q21)$.²⁷

Clinical Features and Prognosis. The gastrointestinal tract is the most common site for extranodal marginal zone lymphoma. The lung, thyroid, ocular adnexa, and breast are other primary sites of involvement. In most cases, the disease is localized to a primary site and regional lymph nodes. Bone marrow is less frequently involved than in other types of indolent lymphoma.²⁸ In cases of gastric MALT lymphoma positive for *H. pylori*, the antibiotic treatment of the infection may induce a remission of the associated lymphoma. Other cases may benefit from local therapy.

Plasma Cell Neoplasms

Definition. Plasma cell neoplasms are characterized by a monoclonal proliferation of terminally differentiated B cells

(i.e., plasma cells). These disorders can present as a localized or disseminated process most commonly involving bone marrow and bone. The clinical features and the primary site of involvement define distinct clinicopathologic entities (Table 36-3).

Plasma cell myeloma is a multifocal accumulation of malignant plasma cells in bone marrow presenting as lytic bone lesions. In most cases, monoclonal immunoglobulin produced by neoplastic plasma cells is detected in serum, urine, or both (monoclonal gammopathy). The overt disease may be preceded by an asymptomatic period of monoclonal gammopathy with only mild bone marrow plasmacytosis (fewer than 10% plasma cells). Approximately 25% of asymptomatic patients with clonal serum immunoglobulin progress to symptomatic plasma cell myeloma.²⁹ The term *monoclonal gammopathy of undetermined significance* (MGUS) is used to encompass the entire patient population with clonal serum immunoglobulin and only mild marrow plasmacytosis. Plasmacytoma, a localized form of plasma cell neoplasm, may present as a solitary bone lesion or involve an extraosseous or extramedullary site, most commonly the nasopharynx, oropharynx, or sinuses.

Morphology and Immunophenotype. Plasma cell myeloma is characterized by marked bone marrow plasmacytosis. Large aggregates and sheets of plasma cells, frequently with cytologic atypia, are present and often constitute more than 30% of marrow cellularity (Figure 36-18, A). Atypical cytologic features seen in plasma cell myeloma include a high nuclear-to-cytoplasmic ratio, dispersed chromatin pattern, and distinct nucleoli (Figure 36-18, B). These changes are rarely seen in reactive conditions and MGUS. Similarly, in reactive plasmacytosis associated with infections and autoimmune disorders,

TABLE 36-3 Clinicopathologic Features of Selected Plasma Cell Neoplasms

Plasma Cell Disorder	Defining Features
Plasma cell myeloma	<ul style="list-style-type: none"> Monoclonal protein in serum or urine Clonal plasma cells in bone marrow or presence of plasmacytoma Organ or tissue impairment (CRAB: hypercalcemia, renal insufficiency, anemia, bone lesions)
Plasma cell leukemia	<ul style="list-style-type: none"> Clonal plasma cell population in bone marrow and other features of plasma cell myeloma in conjunction with peripheral blood involvement: $>2 \times 10^9/L$ or $>20\%$ circulating plasma cells May present with involvement of spleen, liver, and body cavity fluid including cerebrospinal fluid
Monoclonal gammopathy of undetermined significance	<ul style="list-style-type: none"> Bone marrow plasmacytosis ($<10\%$ plasma cells) Monoclonal gammopathy (<30 g/L) No lytic bone lesions or organ/tissue impairment
Solitary plasmacytoma of bone	Localized bone mass composed of plasma cells
Extraosseous plasmacytoma	Localized extraosseous mass composed of plasma cells

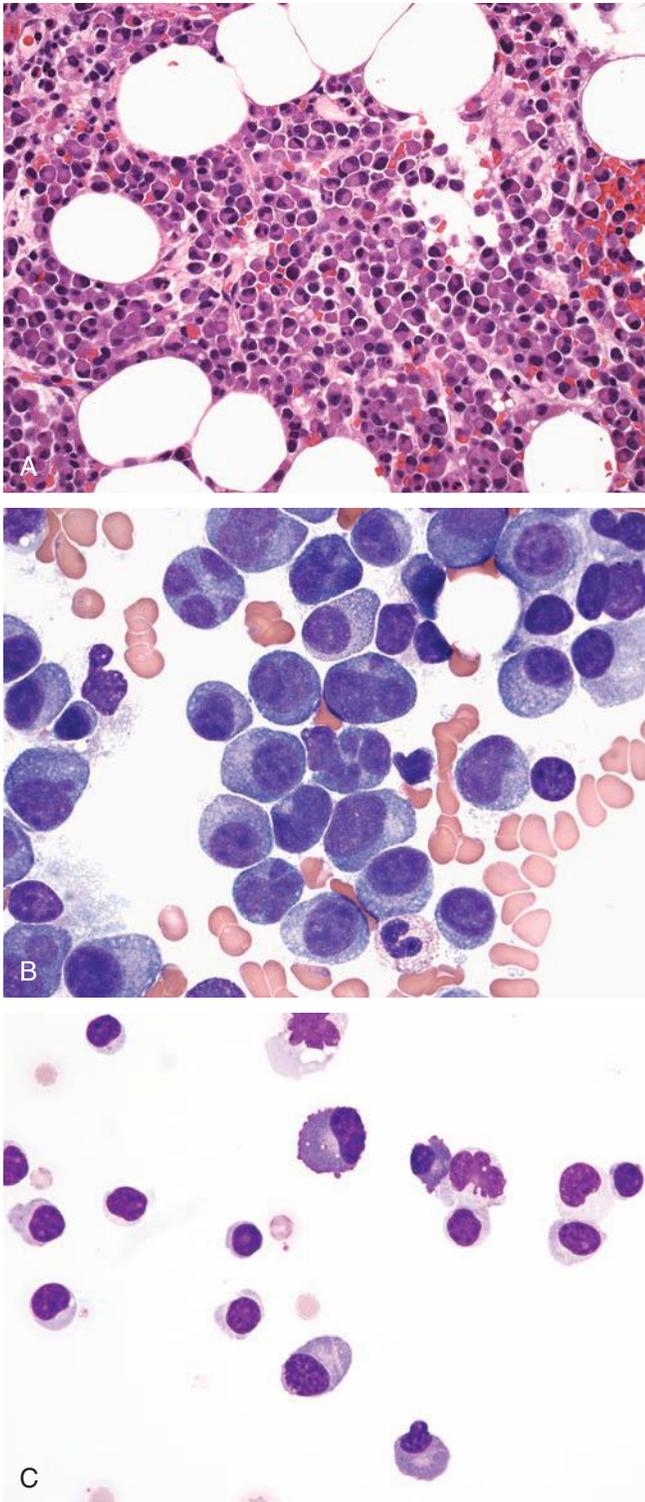


Figure 36-18 Plasma cell myeloma. **A**, Bone marrow biopsy specimen showing large aggregates of plasma cells (hematoxylin and eosin stain, $\times 400$). **B**, Neoplastic plasma cells with cytotypic atypia (bone marrow, Wright-Giemsa stain, $\times 1000$). **C**, Plasma cells in the pleural fluid of a patient with plasma cell myeloma (Wright-Giemsa stain, $\times 1000$).

plasma cells appear scattered throughout the marrow and form small clusters around the vessels. Large aggregates and sheets of plasma cells, which are commonly seen in plasma cell myeloma, are not present in reactive conditions. Rarely, patients with plasma cell myeloma show a marked increase in circulating plasma cells. The term *plasma cell leukemia* is reserved for cases with more than 20% circulating plasma cells or plasma cell counts exceeding $2 \times 10^9/L$. Neoplastic plasma cells can also infiltrate spleen, liver, or lymph nodes and involve body cavity fluids (Figure 36-18, C). Plasmacytoma represents a localized, mass-forming, monoclonal plasma cell proliferation.

Neoplastic plasma cells show an immunophenotype similar to that of their normal counterparts. At this terminal stage of differentiation, pan-B cell markers CD19 and CD20 and surface immunoglobulin chains are usually absent. Plasma cells are positive for CD138 (syndecan-1), high-density CD38 antigen, and monoclonal cytoplasmic immunoglobulins. These monoclonal proteins, in the form of complete immunoglobulin—most commonly IgG or IgA or isolated clonal light chains—are secreted by the neoplastic plasma cells and are seen in serum and urine as monoclonal spikes. In addition, neoplastic plasma cells can show an expression of other antigens typically not expressed by normal plasma cells, such as CD56 and myeloid markers.

Clinical Features and Prognosis. Plasma cell myeloma is a disease of older individuals (median age, 70 years).³⁰ Bone pain and pathologic fractures are directly related to the proliferation of neoplastic plasma cells. These cells produce factors that cause localized bone destruction (lytic lesions on radiographic examination) and hypercalcemia. Renal insufficiency is triggered by the obstruction or direct damage of renal tubules by monoclonal protein. Cytopenias are related to the replacement of normal trilineage hematopoiesis by massive plasma cell infiltrates. Depressed normal immunoglobulin levels result in a susceptibility to infections, which commonly occur in patients with plasma cell myeloma. High levels of serum immunoglobulins also may interfere with the coagulation cascade and impair circulation through an increase in serum viscosity. Tissue deposits of clonal immunoglobulins, called *amyloidosis*, may compromise kidney, heart, and liver function and cause peripheral neuropathies.

Most of the cases show a rapidly progressive course, and the median survival is 3 years. The prognosis is closely related to the number of plasma cells in the bone marrow and to clinical features reflecting overall tumor burden. Patients with more than 50% bone marrow plasma cells, associated renal failure, and severe anemia have a shorter survival than patients with fewer than 20% plasma cells and preserved renal function.

Typically, patients with bone and extraosseous plasmacytomas are younger and respond favorably to local radiation therapy. Approximately 15% progress to plasma cell myeloma.

Asymptomatic monoclonal gammopathy (MGUS) occurs in fewer than 5% of individuals older than 70 years.²⁹ As discussed earlier, 25% of patients with MGUS develop overt myeloma. That is why all individuals with MGUS should be monitored closely with repeat measurements of serum immunoglobulin levels and bone marrow examinations.

Diffuse Large B Cell Lymphoma

Definition. The defining feature of DLBCL is the large cell size. In contrast to the neoplastic cells in the lymphoproliferative disorders discussed so far, DLBCL cells are significantly larger than normal lymphocytes. Most show a diffuse histologic growth pattern and can differ significantly in cytologic appearance and immunophenotype. DLBCL is one of the most common lymphomas, accounting for 30% to 40% of all non-Hodgkin lymphoma cases.⁹

Morphology and Immunophenotype. The most common type, DLBCL not otherwise specified, shows a diffuse proliferation of large lymphoid cells replacing normal nodal architecture. Cells are at least twice the size of normal small lymphocytes and show single or multiple nucleoli and ample cytoplasm (Figure 36-19). In rare cases, in addition to neoplastic large lymphoid cells, there is a considerable admixture of background histiocytes and small lymphocytes.

As in other B cell lymphomas, pan-B cell antigens are expressed. DLBCL can originate from a variety of stages in B cell development—hence the coexpression of other markers is heterogeneous. CD5, CD10, BCL6, CD30, and CD138 can be present (Table 36-2).

Clinical Features and Prognosis. Although, as with most lymphomas, the median age at diagnosis is in the sixties, DLBCL can be seen in children and young adults. Most commonly, it presents as a localized disease involving a group of lymph nodes. Bone marrow involvement is rare at presentation but can occur later in the course of the disease. DLBCL can also be seen in extranodal sites, including the gastrointestinal tract, central nervous system, bone, and serous effusions. DLBCL is an aggressive neoplasm with a proliferation rate frequently exceeding 40%, which makes it more sensitive to multiagent chemotherapy. The prognosis depends on a variety of clinical parameters, such as patient age, the extent of disease, and the site of involvement. The original morphologic subclassification of DLBCL was flawed by poor intraobserver and interobserver

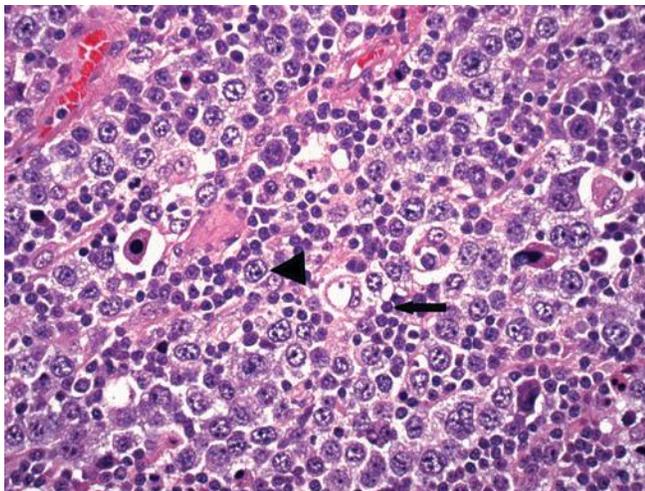


Figure 36-19 Diffuse large B cell lymphoma with proliferation of large lymphoid cells. Note the size difference between small lymphocytes (*arrow*) and neoplastic B cells (*arrowhead*) (hematoxylin and eosin stain, $\times 500$).

reproducibility.³¹ However, recent results of gene microarray studies have shown that patients with DLBCL of follicle center origin have better survival than patients with other subtypes.³²

Burkitt Lymphoma

Definition. Burkitt lymphoma is characterized by medium-sized, highly proliferating lymphoid cells with basophilic vacuolated cytoplasm. The WHO classification lists three variants of this lymphoma: endemic (occurring predominantly in Africa), sporadic, and immunodeficiency associated.

Morphology and Immunophenotype. The lymphoid proliferation is diffuse and at low magnification shows a prominent “starry sky” pattern imparted by numerous tingible-body macrophages (Figure 36-20, A). The macrophages are responsible for phagocytosing apoptotic debris, a by-product of the extremely high proliferative activity. Lymphoma cells are medium size with round nuclei, finely distributed chromatin, and small nucleoli. The cytoplasm is deeply basophilic and highly vacuolated, a feature best displayed on touch imprints or other cytologic preparations (Figure 36-20, B).

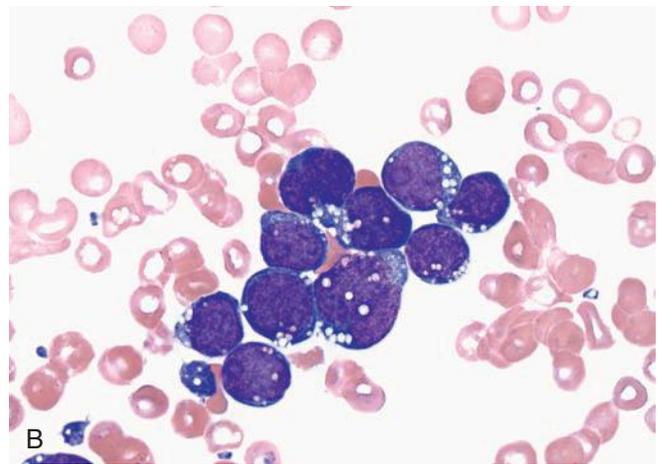
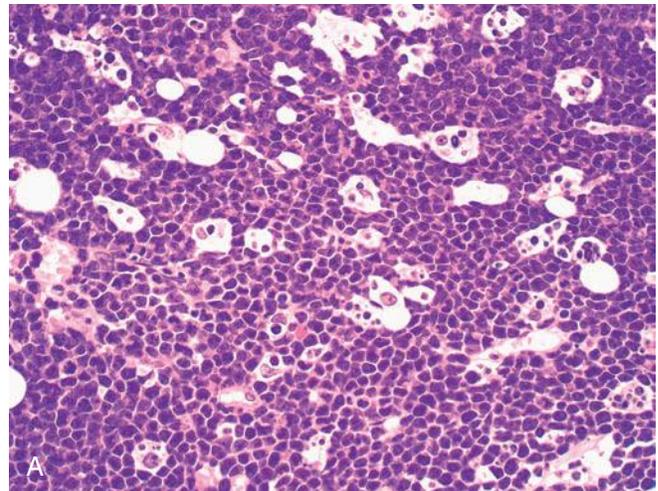


Figure 36-20 Burkitt lymphoma. **A**, “Starry sky” pattern imparted by numerous macrophages with apoptotic debris (hematoxylin and eosin stain, $\times 400$). **B**, Touch preparation showing characteristic cells of Burkitt lymphoma. Note the deeply basophilic cytoplasm with numerous vacuoles (Wright-Giemsa stain, $\times 1000$).

The immunophenotype of Burkitt lymphoma reflects germinal center origin. CD19, CD20, CD10, and BCL6 antigens are present. There is surface expression of monoclonal immunoglobulin light chains. BCL2 is absent. The hallmark of Burkitt lymphoma is a high proliferation rate. Nearly 100% of Burkitt lymphoma cells are actively proliferating. This feature is linked to the constitutive expression of *MYC* gene (cell cycle gatekeeping gene) secondary to its translocation under the promoter of immunoglobulin heavy or light chain genes [t(8;14), t(2;8), or t(8;22)]. This translocation is pathognomonic for Burkitt lymphoma, and its demonstration is required for a definitive diagnosis.

Clinical Features and Prognosis. The clinical presentation of Burkitt lymphoma is dependent on the variant (endemic, sporadic, or immunodeficiency associated). The endemic form presents in young children (4 to 7 years of age), most commonly as a jawbone mass. The sporadic variant, seen in the United States and Europe, occurs in children and young adults most commonly as an abdominal mass. Gastrointestinal tract and abdominal lymph nodes are often involved. Other extranodal sites, such as gonads and breasts, can be a site of primary disease. Immunodeficiency-associated Burkitt lymphoma presents most often as nodal disease. Independent of the presentation, Burkitt lymphoma commonly involves the central nervous system, bone marrow, and peripheral blood (Burkitt leukemia). Epstein-Barr virus (EBV) is present in a proportion of patients.

A diagnosis of Burkitt lymphoma is a medical emergency. Because of its high proliferation rate, the doubling time is extremely short. The chemotherapy is significantly different from that used for other types of high-grade lymphoma. These highly aggressive treatment regimens take into account the high proliferative activity and contribute to high cure rates for childhood and adult Burkitt lymphoma: 60% to 90%, depending on the stage of the disease.^{33,34} Immunodeficiency-associated Burkitt lymphoma occurs predominantly in HIV-positive patients. Its prognosis is not as favorable as for other variants.

Mature T Cell and Natural Killer Cell Lymphomas

Lymphomas derived from mature T cells and natural killer cells are much less common than the previously discussed mature B cell neoplasms and account for approximately 10% of all lymphomas. The incidence of the specific subtypes of T cell lymphoma shows geographic and ethnic variability. In certain geographic regions, T cell malignancies may be more prevalent than in the United States. Compared with B cell lymphomas, T cell neoplasms occur more frequently in extranodal sites. The most common skin lymphoma, mycosis fungoides, is of T cell phenotype.

Although morphology is an important criterion in the diagnosis of T cell lymphomas, a significant morphologic and cytologic variability is seen within specific subtypes. Similarly, the immunophenotypic features are not as specific as those seen in B cell malignancies. Due to the significant morphologic and immunophenotypic variability, an integration of morphologic, immunophenotypic, cytogenetic, molecular, and clinical

information, as recommended by the WHO classification, is crucial in diagnosing T cell and natural killer cell malignancies. Until recently, the demonstration of clonality in T cell proliferations was limited to molecular methods showing T cell receptor gene rearrangements. The development of multiple antibodies directed against the variable region of the T cell receptor enables the determination of T cell clonality by flow cytometry.³⁵

Mycosis Fungoides and Sézary Syndrome

Definition. Mycosis fungoides is the most common cutaneous lymphoma. It is composed of small to medium-sized lymphoid cells with irregular nuclear outlines (cerebriform nuclei). These cells show a predilection for the epidermis (epidermotropism) and dermis and may spread to regional lymph nodes. Sézary syndrome presents as a disseminated disease with widespread skin involvement (erythroderma), lymphadenopathy, and circulating lymphoma cells (Sézary cells with characteristic cerebriform nuclei) (Figure 36-21, A).

Morphology and Immunophenotype. In mycosis fungoides, the extent of cutaneous infiltrate is related to the stage of the disease. Early lesions show patchy or lichenoid infiltrate of the dermis by small to medium-sized lymphoid cells with irregular nuclear outlines (Figure 36-21, B). The aggregates of neoplastic lymphocytes in epidermis, called Pautrier microabscesses, are frequently seen in mycosis fungoides (Figure 36-21, C). Later in the course of the disease, cutaneous infiltrates may become more dense and form tumor-like lesions. The involvement of regional lymph nodes and peripheral blood may be present, especially in advanced stages of the disease.

The immunophenotype is similar to that of T lymphocytes normally present in the skin. The expression of pan-T cell markers CD3, CD5, and CD2 is seen along with CD4 antigen. An important feature, rarely seen in benign lymphoid infiltrates, is the absence of CD7 antigen. The T cell receptor gene is clonally rearranged.

Sézary syndrome is by definition a disseminated disease with leukemic presentation and skin and lymph node involvement.^{36,37} The malignant cells are medium size with cerebriform nuclei. Skin and lymph node involvement is more pronounced than in early stages of mycosis fungoides and shows diffuse, monotonous lymphoid infiltrates. Due to low interobserver reproducibility, the determination of peripheral blood involvement based purely on morphology is not recommended. Currently, both morphologic and immunophenotypic evaluations are performed in order to demonstrate at least 1000 Sézary cells/ μ L, a CD4-to-CD8 ratio of more than 10 (due to significant numbers of circulating CD4⁺ Sézary cells), and an aberrant immunophenotype.¹ The latter is defined as a significant loss of CD7, CD26, or T cell marker(s) on CD4⁺ T cells.

Clinical Features and Prognosis. The incidence of mycosis fungoides increases with age, and the average age at presentation is 55 to 60 years. The survival of patients with early-stage disease is excellent, because progression and development of disseminated lymphoma are very slow.³⁶ In

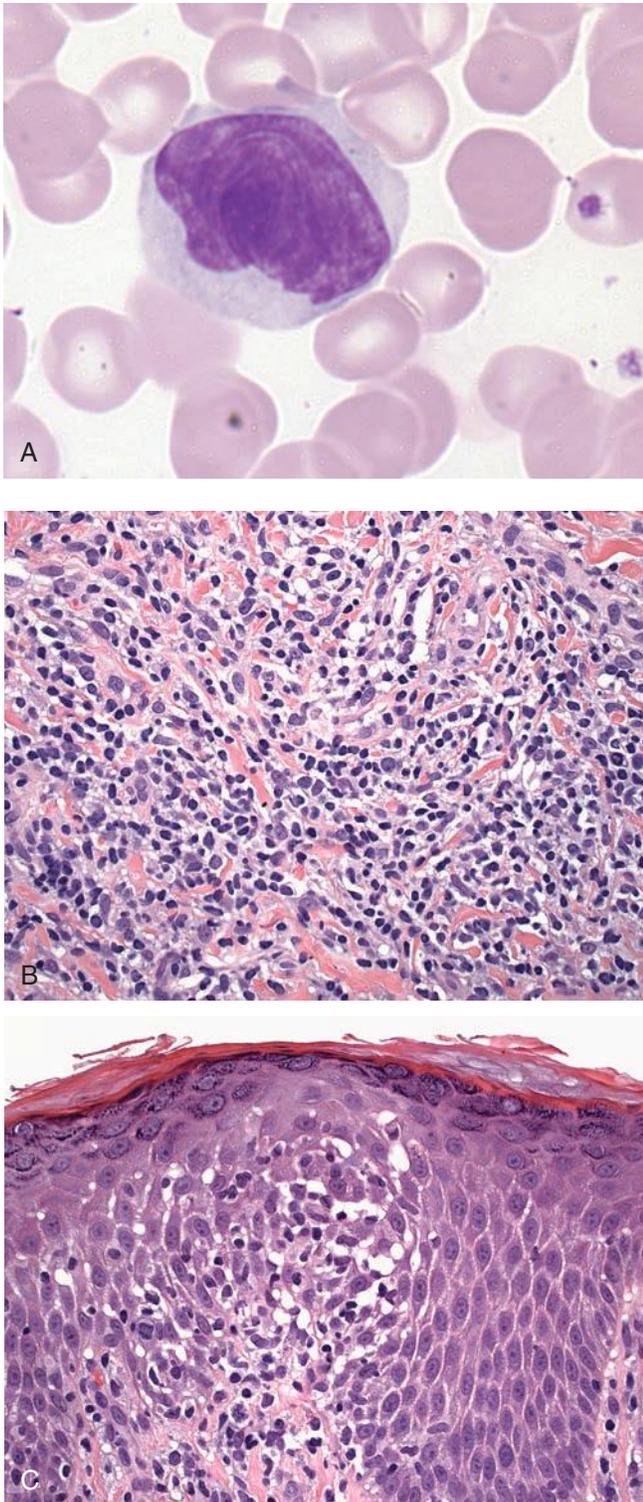


Figure 36-21 Mycosis fungoides. **A**, Sezary cell (Wright-Giemsa stain, $\times 1000$) **B**, Dermal infiltrate of medium-sized lymphoid cells with angulated nuclei (hematoxylin and eosin stain, $\times 400$). **C**, Neoplastic lymphocytes invading the epidermis (hematoxylin and eosin stain, $\times 400$).

this patient group, 10-year disease-specific survival was reported as 97% to 98%. In most cases, only local treatment is necessary. In contrast, Sézary syndrome is an aggressive lymphoma with a low (10% to 20%) 5-year survival rate.³⁷

Peripheral T Cell Lymphoma, Unspecified

Definition. Peripheral T cell lymphoma, unspecified, comprises a morphologically heterogeneous group of lymphomas with mature T cell phenotype.

Morphology and Immunophenotype. Lymph node involvement is usually diffuse with a prominent vascular proliferation. The cytologic features are variable with medium-sized to large cells with atypical and occasionally pleomorphic nuclei (Figure 36-22). Certain variants show a considerable admixture of reactive small lymphocytes, immunoblasts, histiocytes, and eosinophils. Bone marrow is frequently involved, but a significant circulating lymphoma component is rarely seen at presentation. Most cases are derived from CD4⁺ T cells and retain this immunophenotype. Variable loss of pan-T cell antigens, including CD7, is seen.

Clinical Features and Prognosis. Peripheral T cell lymphoma is an aggressive disease occurring predominantly in older adults (average age of 60 years). Generalized lymphadenopathy and a variety of constitutional symptoms, such as fever, night sweats, weight loss, and pancytopenia, are present at diagnosis. The 3-year survival rate is reported to be approximately 40%.³⁸ Biologic features and advanced stage at presentation contribute to the dismal prognosis. In addition, few treatment regimens have been developed specifically for T cell lymphomas; aggressive B cell lymphoma protocols are commonly used to treat these disorders.

Anaplastic Large Cell Lymphoma

Definition. Although considerable morphologic variability can be seen, a typical case of anaplastic large cell lymphoma

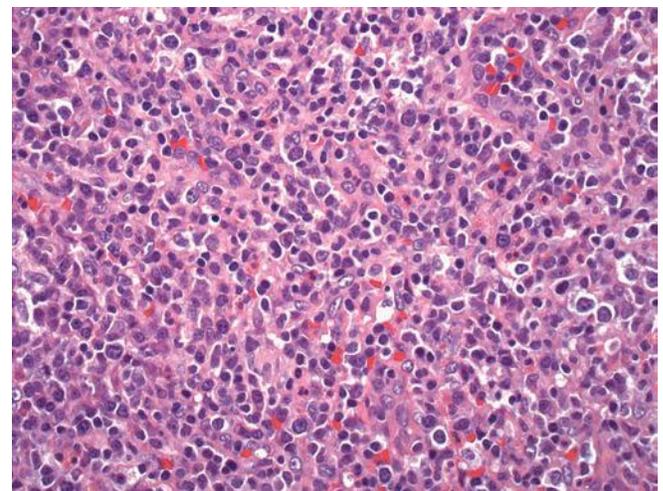


Figure 36-22 Peripheral T cell lymphoma showing heterogeneous population of small, medium-sized, and large lymphoid cells (hematoxylin and eosin stain, $\times 500$).

is characterized by large atypical cells with pleomorphic nuclei and abundant cytoplasm. The expression of CD30 antigen and ALK-1 protein is seen in the majority of cases.

Morphology and Immunophenotype. Numerous morphologic variants have been described, depending on the predominant architectural and cytologic features. The lymph node architecture is most often diffusely effaced by malignant lymphoid cells (Figure 36-23, A). Occasionally, lymph node involvement may be partial with characteristic infiltrates of nodal sinuses. When significant fibrosis is present, anaplastic large cell lymphoma may resemble classical Hodgkin lymphoma. Regardless of the histologic variant, in almost every case, at least a proportion of cells are large with abundant cytoplasm and pleomorphic, eccentric, kidney-shaped nuclei, so-called hallmark cells. Leukemic involvement is not frequent, but it may be seen in the small cell variant. In such cases, the peripheral blood film shows atypical lymphoid cells with indented nuclei reminiscent of Sézary cells (Figure 36-23, B). Immunophenotyping is instrumental for prompt diagnosis.

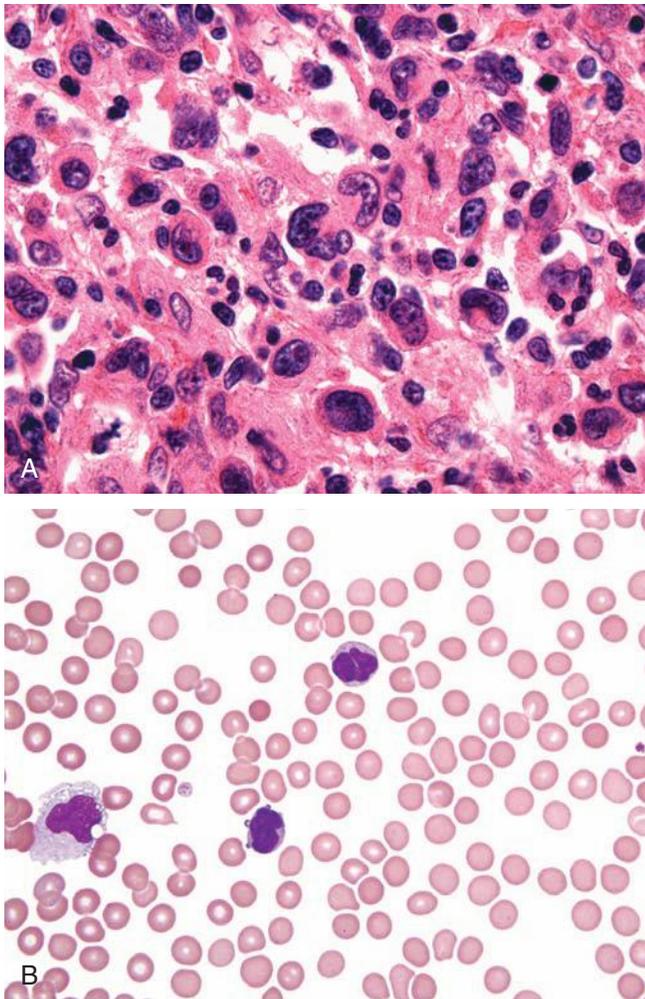


Figure 36-23 A, Anaplastic large cell lymphoma with large pleomorphic cells (hematoxylin and eosin stain, $\times 1000$). B, Peripheral blood involvement by small cell variant of anaplastic large cell lymphoma (Wright-Giemsa stain, $\times 1000$).

CD30 antigen and, in most cases, ALK-1 protein are defining immunophenotypic features of this lymphoma. The overexpression of ALK-1 is most often due to t(2;5)(p23;35) between the *ALK-1* and nucleophosmin genes. Alternative partner genes for *ALK-1* translocation have also been identified. The cytotoxic T cell origin of this lymphoma can be demonstrated by immunohistochemistry showing expression of cytotoxic antigens such as TIA-1, granzyme, and perforin.³⁹ Pan-T cell markers (CD3, CD7, CD5) are often absent. The most commonly expressed T cell lineage-associated antigens are CD4 and CD2. Pan-hematopoietic antigen CD45 is expressed only in a proportion of cases (Table 36-4). By flow cytometry, the coexpression of myeloid markers such as CD13, CD33, and CD15 is seen in approximately 50% of cases. In cases negative for the T cell-associated antigens and ALK-1 protein, the demonstration of clonal T cell receptor gene rearrangement can help to render a definitive diagnosis.

Clinical Features and Prognosis. Anaplastic large cell lymphoma is less frequent in adults. However, it is one of the most common lymphomas in the pediatric population, representing 10% to 15% of childhood lymphomas. Anaplastic large cell lymphoma presents as disseminated nodal disease with constitutional symptoms. Extranodal sites, including skin, can also be involved. The most important prognostic feature is the expression of ALK-1 protein. ALK-1⁺ disease has a favorable prognosis, whereas ALK-1⁻ disease shows survival rates more comparable to those of peripheral T cell lymphoma, unspecified.

Hodgkin Lymphoma

Hodgkin lymphoma can be divided into two broad categories: nodular lymphocyte-predominant Hodgkin lymphoma and classical Hodgkin lymphoma.¹ Although both disorders occur preferentially in young individuals and share certain morphologic characteristics, more recent studies have shown that they are biologically distinct entities, and they are discussed separately.

Nodular Lymphocyte-Predominant Hodgkin Lymphoma

Definition. Nodular lymphocyte-predominant Hodgkin lymphoma is a B cell neoplasm composed of relatively rare

TABLE 36-4 Immunophenotypic Features of Lymphomas Composed of Large Lymphoid Cells

Antigen	NLPHL	Classical HL	DLBCL	ALCL
CD30	—	+	+/-	+
CD15	—	+	—	—
CD45	+	—	+	+/-
CD20	+	+/-*	+	—
CD3	—	—	—	+/-†

ALCL, Anaplastic large cell lymphoma; DLBCL, diffuse large B cell lymphoma; HL, Hodgkin lymphoma; NLPHL, nodular lymphocyte-predominant Hodgkin lymphoma.

*If positive, the immunoreactivity is weak and present only in a proportion of neoplastic cells.

†Other T cell markers, such as CD2 and CD4, are more often present.

neoplastic cells (lymphocytic/histiocytic or “popcorn” cells) scattered within nodules of reactive lymphocytes.

Morphology and Immunophenotype. The normal architecture of a lymph node is replaced by a nodular proliferation of small lymphocytes and scattered lymphocytic/histiocytic or popcorn cells, the latter being the neoplastic cells of nodular lymphocyte-predominant Hodgkin lymphoma (Figure 36-24). These are large lymphoid cells with abundant cytoplasm and vesicular multilobated nuclei (popcorn nuclei). The nucleoli are inconspicuous.

The lymphocytic/histiocytic cells are of follicle center cell origin and are positive for B cell markers, including CD20 antigen, BCL6, and immunoglobulin chains (Table 36-4).⁴⁰ The neoplastic cells do not show evidence of EBV infection. In addition to neoplastic cells, the nodules are composed of CD20⁺ small B cells, T lymphocytes, and CD21 positive follicular dendritic cell meshworks.

Clinical Features and Prognosis. Most patients are males in their thirties and present with localized peripheral lymphadenopathy. Mediastinal lymph node involvement is rare. As for classical Hodgkin lymphoma, the prognosis is excellent, with survival rates of 80% to 90% when the disease is diagnosed in the early stages.⁴¹

Classical Hodgkin Lymphoma

Definition. Classical Hodgkin lymphoma comprises a heterogeneous group of lymphoid neoplasms derived from the germinal center.⁴² It is characterized by the presence of relatively few diagnostic neoplastic cells, Reed-Sternberg cells, in a rich reactive background. The incidence of this disease varies in different geographic regions. In the United States and Europe, it is a common form of lymphoma occurring in young adults. In the United States, approximately 7400 new cases are diagnosed annually.⁵ A bimodal age distribution is observed,

with incidence peaks between 15 and 34 years and older than 54 years.

Morphology and Immunophenotype. On the basis of architectural features, the composition of the reactive background, and relative proportion of neoplastic cells, classical Hodgkin lymphoma can be divided into four subtypes (Table 36-5):

1. Nodular sclerosis
2. Mixed cellularity
3. Lymphocyte rich
4. Lymphocyte depleted

Reed-Sternberg cells are present in all subtypes of classical Hodgkin lymphoma. The typical Reed-Sternberg cell is large with a bilobed nucleus or two nuclei with prominent eosinophilic nucleoli and abundant cytoplasm (Figure 36-25, A). When encountered in an appropriate background, Reed-Sternberg cells are pathognomonic for the diagnosis. However, not all neoplastic cells of classical Hodgkin lymphoma show the typical morphology of Reed-Sternberg cells. Variants of neoplastic cells, including Hodgkin cells, mummified cells, and lacunar cells, are often encountered in a single lymph node. Hodgkin cells are large mononuclear lymphoid cells with an oval nucleus, thick nuclear membrane, distinct eosinophilic nucleolus, and abundant cytoplasm. Mummified cells are degenerated or apoptotic cells with a pyknotic nucleus and condensed cytoplasm. Lacunar cells occur predominantly in the nodular sclerosis variant of classical Hodgkin lymphoma and are characterized by a lobated nucleus and artifactual retraction of cytoplasm secondary to formalin fixation. Because of this artifact, the cells appear to be situated in a clear space (i.e., lacuna).

In all subtypes of classical Hodgkin lymphoma, Reed-Sternberg cells and their variants have a similar immunophenotype (Table 36-5). They are CD30⁺ in all cases (Figure 36-25, B) and CD15⁺ in approximately 80% of cases. The CD15

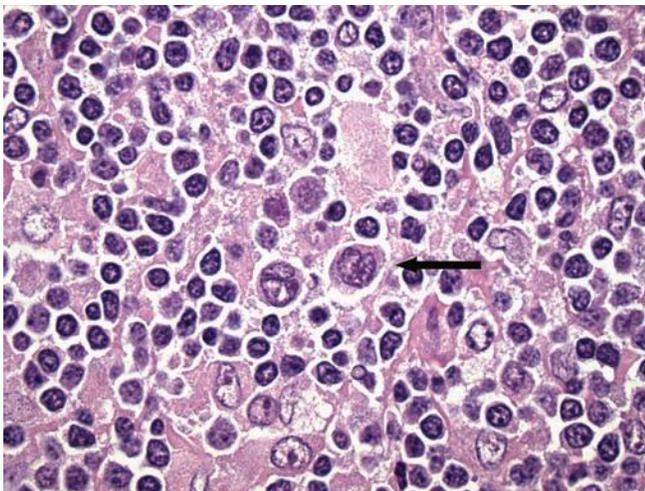


Figure 36-24 Characteristic popcorn (lymphocytic/histiocytic) cells of nodular lymphocyte-predominant Hodgkin lymphoma (arrow) (hematoxylin and eosin stain, $\times 1000$).

TABLE 36-5 Morphologic Subtypes of Classical Hodgkin Lymphoma

Subtype	Neoplastic Cells	Additional Morphologic Features
Nodular sclerosis	RS cells; Hodgkin cells; lacunar cells	Fibrotic bands; background of small lymphocytes, histiocytes, and eosinophils
Mixed cellularity	RS cells; Hodgkin cells	Background of small lymphocytes, eosinophils, neutrophils, histiocytes, plasma cells; no fibrotic bands
Lymphocyte rich	RS cells; Hodgkin cells	Diffuse nodular background of small lymphocytes; no or few eosinophils and neutrophils
Lymphocyte depleted	RS cells; Hodgkin cells	Numerous RS cells and Hodgkin lymphoma cells; few background lymphocytes

RS, Reed-Sternberg.

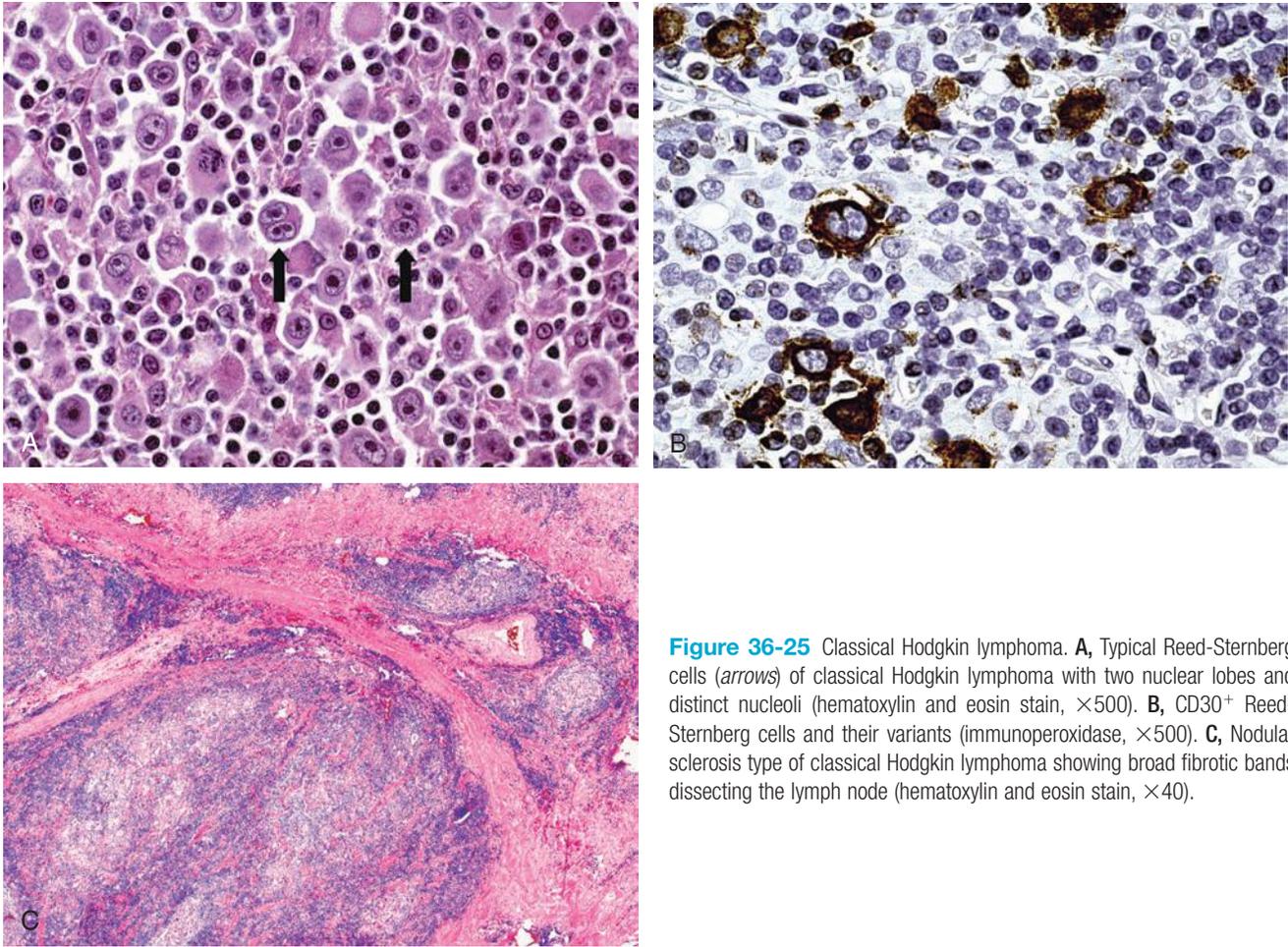


Figure 36-25 Classical Hodgkin lymphoma. **A**, Typical Reed-Sternberg cells (*arrows*) of classical Hodgkin lymphoma with two nuclear lobes and distinct nucleoli (hematoxylin and eosin stain, $\times 500$). **B**, CD30⁺ Reed-Sternberg cells and their variants (immunoperoxidase, $\times 500$). **C**, Nodular sclerosis type of classical Hodgkin lymphoma showing broad fibrotic bands dissecting the lymph node (hematoxylin and eosin stain, $\times 40$).

immunoreactivity may be weak and seen in only a few malignant cells. The expression of B cell marker CD20 is weak to absent. Similarly, CD45 antigen is absent. The frequency of EBV infection depends on the subtype of classical Hodgkin lymphoma. The background small lymphocytes are predominantly CD4⁺ T cells.

Nodular Sclerosis Classical Hodgkin Lymphoma. The defining feature of the nodular sclerosis subtype is the presence of broad collagen bands transecting the lymph node and thickening of nodal capsule (Figure 36-25, C). The background cellularity includes small lymphocytes, eosinophils, and histiocytes. This is the most common subtype of classical Hodgkin lymphoma, accounting for 70% of cases. The frequency of immunohistochemically demonstrable EBV infection is lowest in this variant.

Mixed Cellularity Classical Hodgkin Lymphoma. In the mixed cellularity subtype, Reed-Sternberg cells and their variants are scattered among the diffuse background composed of small lymphocytes, histiocytes, eosinophils, neutrophils, and plasma cells. Typical Reed-Sternberg cells, mononuclear Hodgkin cells, and mummified cells are seen; however, lacunar cells are absent. Similarly, fibrotic bands and capsular thickening are not present. Approximately 20% of classical Hodgkin

lymphomas show this morphology. An association with EBV infection is seen in 75% of cases.

Lymphocyte-Rich Classical Hodgkin Lymphoma. In the lymphocyte-rich subtype, scattered mononuclear Hodgkin and Reed-Sternberg cells are seen together with a vaguely nodular background of small lymphocytes. Nodules represent remnants of mantle zones and germinal centers. Compared with other subtypes of classical Hodgkin lymphoma, the background cellularity is less heterogeneous.

Lymphocyte-Depleted Classical Hodgkin Lymphoma. The lymphocyte-depleted subtype is an uncommon variant of classical Hodgkin lymphoma occurring predominantly in immunodeficient patients. There is a paucity of reactive background, and neoplastic Reed-Sternberg cells and their variants are much more frequent. In most cases, neoplastic cells show evidence of EBV infection.

Clinical Features and Prognosis. With the exception of the lymphocyte-rich variant, which occurs in a slightly older population, classical Hodgkin lymphoma is a disease of young adults with a peak incidence at 15 to 35 years. Mostly peripheral lymph nodes are involved, except in the nodular sclerosis variant, which often shows mediastinal lymphadenopathy.

With the contemporary treatment protocols combining chemotherapy and radiotherapy, the cure rates are 80% to 90%, depending on the stage of the disease, patient age, and clinical symptoms. The best prognosis is seen in the nodular sclerosis subtype. Lymphocyte-depleted Hodgkin lymphoma is the most aggressive variant of classical Hodgkin lymphoma, especially in

HIV-positive patients. In this patient group, Hodgkin lymphoma also may manifest in unusual extranodal sites, including bone marrow. Patients with classical Hodgkin lymphoma treated with a combination of chemotherapy and radiotherapy are at high risk of developing secondary malignancies, including lung and breast carcinomas and acute leukemia.

SUMMARY

- Histologic components of normal lymph nodes include the cortex, paracortex, medullary cords, and sinuses. These are structural and functional compartments from which reactive hyperplasias and neoplasms originate.
- Lymphomas are neoplasms of the lymphoid system arising at specific stages of lymphoid differentiation.
- Modern lymphoma classification incorporates morphologic, immunophenotypic, molecular, laboratory data, and clinical characteristics.
- Lymphomas are broadly divided into neoplasms derived from precursor (immature) and mature lymphoid cells, and B cell and T cell malignancies.
- The most common mature B cell neoplasms are follicular lymphoma and DLBCL.
- The T cell neoplasms most common in the United States and Europe are peripheral T cell lymphoma, unspecified, and anaplastic large cell lymphoma.
- In CLL, the peripheral blood and bone marrow display smudge cells and small lymphoid cells with coarse chromatin, inconspicuous nucleoli, and scant cytoplasm.
- In HCL, small B lymphocytes have abundant cytoplasm and fine cytoplasmic projections.
- Hodgkin lymphoma has been shown to be of B cell origin.
- Hodgkin lymphoma is subclassified based on morphologic and immunophenotypic features.
- B cell and T cell lymphomas and Hodgkin lymphoma involve mainly lymph nodes; the involvement of extranodal sites and bone marrow or peripheral blood occurs with varying frequency.
- In general, lymphomas occur in elderly individuals; however, specific subtypes such as Hodgkin lymphoma show a predilection for younger age groups.
- The prognosis depends on lymphoma subtype. Indolent lymphomas show a protracted course but are largely incurable with current chemotherapeutic regimens. In contrast, aggressive lymphomas have a more rapidly progressive course, and the cure rates are higher.

Now that you have completed this chapter, go back and read again the case study at the beginning and respond to the questions presented.

REVIEW QUESTIONS

Answers can be found in the Appendix.

1. In most cases, the diagnosis of lymphoma relies on all of the following *except*:
 - a. Microscopic examination of affected lymph nodes
 - b. Immunophenotyping using immunohistochemistry or flow cytometry
 - c. Molecular or cytogenetic analysis
 - d. Peripheral blood examination and a complete blood count
2. The most common lymphoma occurring in young adults is:
 - a. Follicular lymphoma
 - b. DLBCL
 - c. Hodgkin lymphoma
 - d. Mycosis fungoides
3. In a normal lymph node, the medulla includes predominantly:
 - a. T cells
 - b. B cells
 - c. Tingible-body macrophages
 - d. Plasma cells
4. The t(11;14) is the defining feature of:
 - a. Follicular lymphoma
 - b. Hodgkin lymphoma
 - c. CLL
 - d. Mantle cell lymphoma
5. The immunophenotype of mycosis fungoides is:
 - a. The normal T cell immunophenotype
 - b. An abnormal T cell immunophenotype with expression of CD4 and loss of CD7 antigen
 - c. A mix of CD4⁺ and CD8⁺ T cells
 - d. An abnormal T cell immunophenotype with expression of CD8 and loss of CD7 antigen
6. What is the major morphologic difference between Hodgkin lymphoma and other B cell lymphomas?
 - a. The extent of the lymph node involvement
 - b. The presence of numerous reactive lymphocytes and only a few malignant cells in Hodgkin lymphoma
 - c. The presence of numerous tingible-body macrophages in Hodgkin lymphoma
 - d. The preservation of normal lymph node architecture in Hodgkin lymphoma

7. Which morphologic diagnosis has to be confirmed with molecular studies demonstrating the presence of t(8;14)?
 - a. Mantle cell lymphoma
 - b. Burkitt lymphoma
 - c. Follicular lymphoma
 - d. Sézary syndrome
8. What is the function of the germinal center?
 - a. Generation of B cells producing immunoglobulins with the highest affinity for a particular antigen through the process of somatic mutation
 - b. Production of plasma cells that secrete specific immunoglobulins following antigenic stimulation
 - c. T cell maturation following T cell education in the thymus
 - d. Generation of dendritic cells with unique antigen-processing capabilities
9. Marked paracortical expansion is most commonly seen in:
 - a. Rheumatoid arthritis
 - b. Syphilis
 - c. Dermatopathic lymphadenopathy
 - d. Follicular lymphoma
10. MGUS is best described as:
 - a. The presence of monoclonal immunoglobulin in serum with only mild bone marrow plasmacytosis
 - b. The presence of monoclonal serum or urine immunoglobulin with significant bone marrow plasmacytosis
 - c. The presence of significant bone marrow plasmacytosis in a patient with only a few clinical symptoms of plasma cell myeloma
 - d. The presence of monoclonal immunoglobulin in a patient with a solitary mass composed of plasma cells

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Normal Hemostasis and Coagulation 37

Margaret G. Fritsma and George A. Fritsma

OUTLINE

Overview of Hemostasis Vascular Intima in Hemostasis

Anticoagulant Properties of Intact Vascular Intima
Procoagulant Properties of Damaged Vascular Intima
Fibrinolytic Properties of Vascular Intima

Platelets

Coagulation System

Nomenclature of Procoagulants
Classification and Function of Procoagulants
Plasma-Based (In Vitro) Coagulation: Extrinsic, Intrinsic, and Common Pathways

Cell-Based (In Vivo, Physiologic) Coagulation

Coagulation Regulatory Mechanisms

Tissue Factor Pathway Inhibitor
Protein C Regulatory System
Antithrombin and Other Serine Protease Inhibitors (Serpins)

Fibrinolysis

Plasminogen and Plasmin
Plasminogen Activation
Control of Fibrinolysis
Fibrin Degradation Products and D-Dimer

OBJECTIVES

After completion of this chapter, the reader will be able to:

1. List the systems that interact to provide hemostasis.
2. Describe the properties of the vascular intima in the initiation and regulation of hemostasis and fibrinolysis.
3. List the hemostatic functions of tissue factor-bearing cells and blood cells, especially platelets, in hemostasis.
4. Describe the relationships among platelet function, von Willebrand factor, and fibrinogen, and their impact on hemostasis.
5. Describe the nature, origin, and function of each of the tissue and plasma factors necessary for normal coagulation.
6. Explain the role of vitamin K in the production and function of the prothrombin group of plasma clotting factors.
7. Distinguish between coagulation pathway serine proteases and cofactors.
8. Describe six roles of thrombin in hemostasis.
9. Diagram fibrinogen structure, fibrin formation, fibrin polymerization, and fibrin cross-linking.
10. For each coagulation complex—extrinsic tenase, intrinsic tenase, and prothrombinase—identify the serine protease and the cofactor forming the complex, the type of cell involved, and the substrate(s) activated.
11. List the factors in order of reaction in the plasma-based extrinsic, intrinsic, and common pathways.
12. Describe the cell-based in vivo coagulation process and the role of tissue factor-bearing cells and platelets.
13. Show how tissue factor pathway inhibitor, the protein C pathway, and the serine protease inhibitor antithrombin function to regulate coagulation and prevent thrombosis.
14. Describe the fibrinolytic pathway, its regulators, and its products.

CASE STUDY

After studying the material in this chapter, the reader should be able to respond to the following case study:

A pregnant woman developed a blood clot in her left leg (deep vein thrombosis, or DVT). Her mother reportedly had a history of thrombophlebitis. She had a brother who was diagnosed with a DVT following a flight from Los Angeles to Sydney, Australia.

1. Is this hemostatic disorder typical of an acquired or an inherited condition?
2. Are these symptoms most likely caused by a deficiency of a procoagulant or an inhibitor?

Hemostasis is a complex physiologic process that keeps circulating blood in a fluid state and then, when an injury occurs, produces a clot to stop the bleeding, confines the clot to the site of injury, and finally dissolves the clot as the wound heals. When hemostasis systems are out of balance, hemorrhage (bleeding) or thrombosis (pathological clotting)

can be life-threatening. The absence of a single plasma procoagulant may destine the individual to lifelong *anatomic hemorrhage*, chronic inflammation, and transfusion dependence. Conversely, absence of a control protein allows coagulation to proceed unchecked and results in thrombosis, stroke, pulmonary embolism, deep vein thrombosis, and cardiovascular events.

Understanding the major systems of hemostasis—blood vessels, platelets, and plasma proteins—is essential to interpreting laboratory test results and to prevent, predict, diagnose, and manage hemostatic disease.

OVERVIEW OF HEMOSTASIS

Hemostasis involves the interaction of vasoconstriction, platelet adhesion and aggregation, and coagulation enzyme activation to stop bleeding. The coagulation system, similar to other humoral amplification mechanisms, is complex because it translates a diminutive physical or chemical stimulus into a profound lifesaving event.¹ The key cellular elements of hemostasis are the cells of the vascular intima, extravascular tissue factor (TF)-bearing cells, and platelets. The plasma components are the coagulation and fibrinolytic proteins and their inhibitors.

Primary hemostasis (Table 37-1) refers to the role of blood vessels and platelets in response to a vascular injury, or to the commonplace *desquamation* of dying or damaged endothelial cells. Blood vessels contract to seal the wound or reduce the blood flow (vasoconstriction). Platelets become activated, adhere to the site of injury, secrete the contents of their granules, and aggregate with other platelets to form a platelet plug. Vasoconstriction and platelet plug formation comprise the initial, rapid, short-lived response to vessel damage, but to control major bleeding in the long term, the plug must be reinforced by fibrin. Defects in primary hemostasis such as collagen abnormalities, thrombocytopenia, qualitative platelet disorders, or von Willebrand disease can cause debilitating, sometimes fatal, chronic hemorrhage.

Secondary hemostasis (Table 37-1) describes the activation of a series of coagulation proteins in the plasma, mostly serine proteases, to form a fibrin clot. These proteins circulate as inactive zymogens (proenzymes) that become activated during the process of coagulation and, in turn, form complexes that activate other zymogens to ultimately generate thrombin, an enzyme that converts fibrinogen to a localized fibrin clot. The final event of hemostasis is fibrinolysis, the gradual digestion and removal of the fibrin clot as healing occurs.²

Although the vascular intima and platelets are associated with primary hemostasis, and coagulation and fibrinolysis are associated with secondary hemostasis, all systems interact in early- and late-hemostatic events. For example, platelets,

although a key component of primary hemostasis, also secrete coagulation factors stored in their granules and provide an essential cell membrane phospholipid on which coagulation complexes form. The remainder of this chapter examines vascular intima, platelets, normal coagulation, coagulation control, and fibrinolysis in detail.

VASCULAR INTIMA IN HEMOSTASIS

The vascular intima provides the interface between circulating blood and the body tissues. The innermost lining of blood vessels is a monolayer of metabolically active *endothelial cells* (EC) (Box 37-1; Figure 37-1).³ Endothelial cells are complex and heterogeneous and are distributed throughout the body. They display unique structural and functional characteristics, depending on their environment and physiologic requirements, not only in subsets of blood vessels such as arteries *versus* veins but also in the various tissues and organs of the body.^{4,5} ECs play essential roles in immune response, vascular permeability, proliferation, and, of course, hemostasis.

ECs form a smooth, unbroken surface that eases the fluid passage of blood. An elastin-rich internal elastic lamina (basement membrane) and its surrounding layer of connective tissues support the ECs. In all blood vessels, fibroblasts occupy the connective tissue layer and produce collagen. Smooth muscle cells in arteries and arterioles, but not in the walls of veins, venules, or capillaries, contract during primary hemostasis.

Anticoagulant Properties of Intact Vascular Intima

Normally, the intact vascular endothelium prevents thrombosis by inhibiting platelet aggregation, preventing coagulation activation and propagation, and enhancing fibrinolysis. Several specific anticoagulant mechanisms prevent intravascular thrombosis (Box 37-2; Figure 37-2). First, ECs are rhomboid and contiguous, providing a smooth inner surface of the blood vessel that prevents harmful turbulence that otherwise may activate platelets and coagulation enzymes. ECs form a physical barrier separating procoagulant proteins and platelets in blood from *collagen* in the internal elastic lamina that promotes platelet adhesion, and *tissue factor* in fibroblasts and smooth muscle cells that activates coagulation.

TABLE 37-1 Primary and Secondary Hemostasis

Primary Hemostasis	Secondary Hemostasis
Activated by desquamation and small injuries to blood vessels	Activated by large injuries to blood vessels and surrounding tissues
Involves vascular intima and platelets	Involves platelets and coagulation system
Rapid, short-lived response	Delayed, long-term response
Procoagulant substances exposed or released by damaged or activated endothelial cells	The activator, tissue factor, is exposed on cell membranes

BOX 37-1 Vascular Intima of the Blood Vessel

Innermost Vascular Lining

Endothelial cells (endothelium)

Supporting the Endothelial Cells

Internal elastic lamina composed of elastin and collagen

Subendothelial Connective Tissue

Collagen and fibroblasts in veins

Collagen, fibroblasts, and smooth muscle cells in arteries

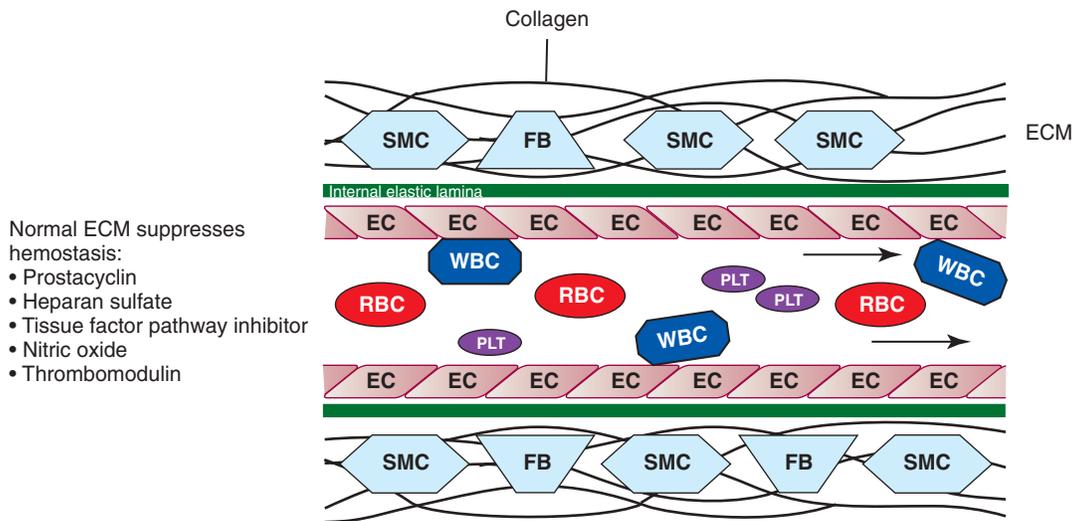


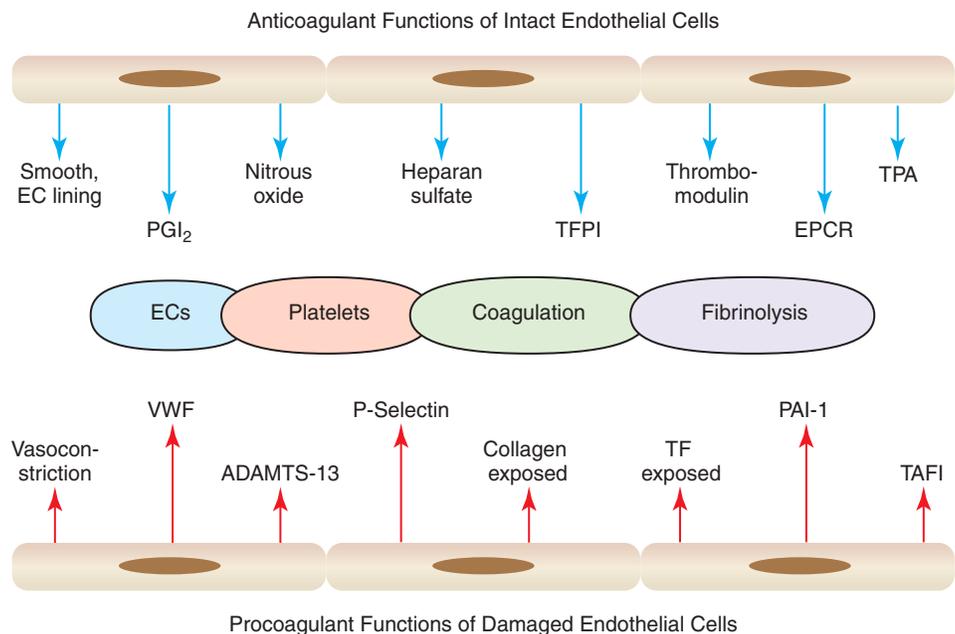
Figure 37-1 Normal blood flow in intact vessels. Smooth, rhomboid endothelial surfaces promote even flow. RBCs and platelets are concentrated toward the center, WBCs roll along the endothelium. The endothelium contains or secretes several hemostasis-suppressing materials. *EC*, Endothelial cell; *ECM*, extracellular matrix; *FB*, fibroblast; *PLT*, platelet; *RBC*, red blood cell; *SMC*, smooth muscle cell; *WBC*, white blood cell; *lines* indicate collagen.

BOX 37-2 Anticoagulant Properties of Intact Endothelium

Composed of rhomboid cells presenting a smooth, contiguous surface
 Secretes the eicosanoid platelet inhibitor *prostacyclin*
 Secretes vascular “relaxing” factor *nitric oxide*
 Secretes the anticoagulant glycosaminoglycan *heparan sulfate*
 Secretes coagulation extrinsic pathway regulator *tissue factor pathway inhibitor*
 Expresses endothelial protein C receptor
 Expresses cell membrane *thrombomodulin*, a protein C coagulation control system activator
 Secretes TPA, thereby activating *fibrinolysis*

ECs synthesize and secrete a variety of substances that maintain normal blood flow. Prostacyclin, a platelet inhibitor and a vasodilator, is synthesized through the eicosanoid pathway (Chapter 13) and prevents unnecessary or undesirable platelet activation in intact vessels.⁶ Nitric oxide is synthesized in ECs, vascular smooth muscle cells, neutrophils, and macrophages. Nitric oxide induces smooth muscle relaxation and subsequent vasodilation, inhibits platelet activation, and promotes angiogenesis and healthy arterioles.^{7,8} An important EC-produced anticoagulant is *tissue factor pathway inhibitor* (TFPI), which controls activation of the tissue factor pathway, also called the extrinsic coagulation pathway.

Figure 37-2 Anticoagulant functions of normal intact endothelial cells and procoagulant properties of endothelial cells when damaged. *EC*, Endothelial cells; *PGI₂*, prostacyclin or prostaglandin I₂; *TFPI*, tissue factor pathway inhibitor; *EPCR*, endothelial cell protein C receptor; *TPA*, tissue plasminogen activator; *VWF*, von Willebrand factor; *ADAMTS-13*, a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13; *TF*, tissue factor; *PAI-1*, plasminogen activator inhibitor-1; *TAFI*, thrombin-activatable fibrinolysis inhibitor.



Finally, ECs synthesize and express on their surfaces inhibitors of thrombin formation, *thrombomodulin*, facilitated by *endothelial protein C receptor (EPCR)*, and *heparan sulfate*. EPCR binds protein C, and thrombomodulin catalyzes the activation of the protein C pathway. The protein C pathway downregulates coagulation by digesting activated factors V and VIII, thereby inhibiting thrombin formation. Heparan sulfate is a glycosaminoglycan that enhances the activity of antithrombin, a serine protease inhibitor.⁹ The pharmaceutical anticoagulant *heparin*, manufactured from porcine gut tissues, resembles EC heparan sulfate in its antithrombin activity. Heparin is used extensively as a therapeutic agent to prevent propagation of the thrombi that cause coronary thrombosis, strokes, deep vein thromboses, and pulmonary emboli.

Procoagulant Properties of Damaged Vascular Intima

Although the intact endothelium has anticoagulant properties, when damaged, the vascular intima promotes coagulation. First, any harmful local stimulus, whether mechanical or chemical, induces vasoconstriction in arteries and arterioles (Table 37-2; Figure 37-2). Smooth muscle cells contract, the vascular lumen narrows or closes, and blood flow to the injured site is minimized. Although veins and capillaries do not have smooth muscle cells, bleeding into surrounding tissues creates extravascular pressure on the blood vessel, effectively minimizing the escape of blood.

Second, the subendothelial connective tissues of arteries and veins are rich in collagen, a flexible, elastic structural protein that binds and activates platelets. Some connective tissue degeneration occurs naturally in aging, which leads to an increased bruising tendency.

Third, ECs secrete von Willebrand factor (VWF) from storage sites called *Weibel-Palade* bodies when activated by vasoactive agents such as thrombin. VWF is a large multimeric glycoprotein that is necessary for platelets to adhere to exposed subendothelial collagen in arterioles.¹⁰

Fourth, on activation, ECs secrete and coat themselves with *P-selectin*, an adhesion molecule that promotes platelet and

leukocyte binding.¹¹ ECs also secrete immunoglobulin-like adhesion molecules called *intercellular adhesion molecules (ICAMs)* and *platelet endothelial cell adhesion molecules (PECAMs)* that further promote platelet and leukocyte binding.¹²

Finally, subendothelial smooth muscle cells and fibroblasts support the constitutive membrane protein tissue factor.¹³ Physiologically, EC disruption exposes tissue factor in subendothelial cells and activates the coagulation system through contact with plasma factor VII. In pathological conditions, tissue factor may also be expressed on bloodborne monocytes during inflammation and sepsis and by tissue factor–positive microparticles derived from membrane fragments of activated or apoptotic vascular cells and possibly on the surface of some ECs.¹⁴ Activation of the TF:VIIa:Xa complex within the circulation is limited by TFPI.

Fibrinolytic Properties of Vascular Intima

ECs support fibrinolysis (Figure 37-2), the removal of fibrin to restore vessel patency, with the secretion of tissue plasminogen activator (TPA). During thrombus formation, both TPA and plasminogen bind to polymerized fibrin. TPA activates fibrinolysis by converting plasminogen to plasmin, which gradually digests fibrin and restores blood flow. ECs also regulate fibrinolysis by providing inhibitors to prevent excessive plasmin generation. ECs, as well as other cells, secrete *plasminogen activator inhibitor 1 (PAI-1)*, a TPA control protein that inhibits plasmin generation and fibrinolysis.¹⁵ Another inhibitor of plasmin generation, *thrombin-activatable fibrinolysis inhibitor (TAFI)*, is activated by thrombin bound to EC membrane thrombomodulin.¹⁶ Elevations in PAI-1 or TAFI can slow fibrinolysis and increase the tendency for thrombosis.

Although the significance of the vascular intima in hemostasis is well recognized, there are few valid laboratory methods to assess the integrity of ECs, smooth muscle cells, fibroblasts, and their collagen matrix.¹⁷ The diagnosis of blood vessel disorders is often based on clinical symptoms, family history, and laboratory tests that rule out platelet or coagulation disorders.

TABLE 37-2 Procoagulant Properties of the Damaged Vascular Intima

Structure	Procoagulant Property
Smooth muscle cells in arterioles and arteries	Induce vasoconstriction
Exposed subendothelial collagen	Binds VWF and platelets
Damaged or activated ECs	Secrete VWF Secrete adhesion molecules: P-selectin, ICAMs, PECAMs
Exposed smooth muscle cells and fibroblasts	Tissue factor exposed on cell membranes
ECs in inflammation	Tissue factor is induced by inflammation

ECs, endothelial cells; ICAMs, intercellular adhesion molecules; PECAMs, platelet endothelial cell adhesion molecules; VWF, von Willebrand factor.

PLATELETS

Platelets are produced from the cytoplasm of bone marrow megakaryocytes (Chapter 13).¹⁸ Although platelets are only 2 to 3 μm in diameter on a fixed, stained peripheral blood film, they are complex, metabolically active cells that interact with their environment and initiate and control hemostasis.¹⁹

At the time of an injury, platelets adhere, aggregate, and secrete the contents of their granules (Table 37-3).^{20,21} *Adhesion* is the property by which platelets bind nonplatelet surfaces such as subendothelial collagen (Figures 37-3 and 37-4). Further, VWF links platelets to collagen in areas of high shear stress such as arteries and arterioles, whereas platelets may bind directly to collagen in damaged veins and capillaries. VWF binds platelets through their glycoprotein GP Ib/IX/V membrane receptor.²² The importance of platelet adhesion is underscored by bleeding disorders such as Bernard-Soulier syndrome, in which the platelet GP Ib/IX/V receptor is absent,

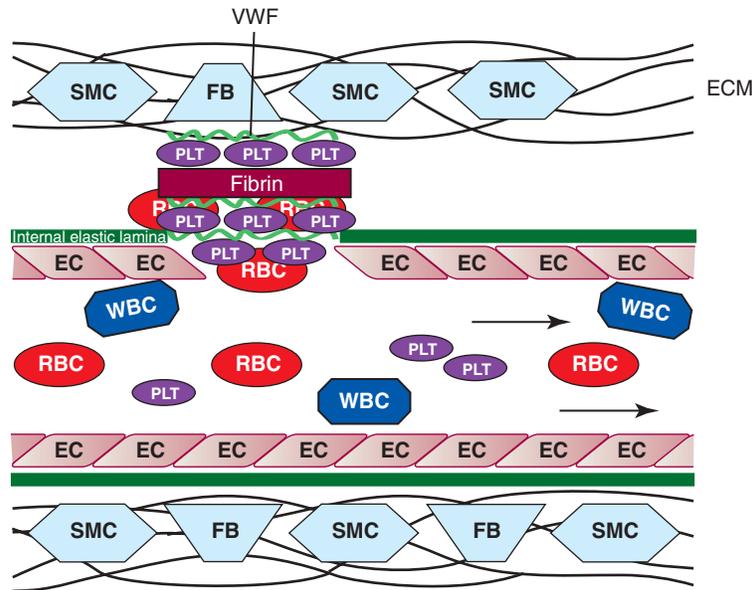


Figure 37-5 Platelet aggregation. In veins and venules, the bulky “red clot” consists of platelets (*PLT*), von Willebrand factor (*VWF*), fibrin, and red blood cells (*RBC*). Though a protective mechanism, the red clot may occlude the vessel, causing venous thromboembolic disease. *EC*, Endothelial cell; *ECM*, extracellular matrix; *FB*, fibroblast; *WBC*, white blood cell; *SMC*, smooth muscle cell; *lines* indicate collagen.

studies, the most commonly used agonists to induce aggregation are thrombin (or thrombin receptor activation peptide, TRAP), arachidonic acid, adenosine diphosphate (ADP), collagen, and epinephrine, which bind to their respective platelet membrane receptors.²⁴

Platelets secrete the contents of their granules during adhesion and aggregation, with most secretion occurring late in the platelet activation process. Platelets secrete procoagulants, such as factor V, VWF, factor VIII, and fibrinogen, as well as control proteins, Ca^{2+} , ADP, and other hemostatic molecules. See [Table 37-4](#) for a summary of the contents of platelet α -granules and dense bodies (dense granules).

During activation, ADP and Ca^{2+} activate phospholipase A_2 , which converts membrane phospholipid to arachidonic acid. *Cyclooxygenase* converts arachidonic acid into prostaglandin endoperoxides. In the platelet, thromboxane synthetase converts

prostaglandins into thromboxane A_2 , which causes Ca^{2+} to be released and promotes platelet aggregation and vasoconstriction ([Figure 37-6](#)). Aspirin acetylation permanently inactivates cyclooxygenase, blocking thromboxane A_2 production and causing impairment of platelet function (aspirin effect).²⁵

Chapter 13 provides an in-depth description of platelet structure and function. Platelet disorders are considered in detail in Chapters 40 and 41.

The platelet membrane is the key surface for coagulation enzyme-cofactor-substrate complex formation.²⁶ Platelets supply Ca^{2+} , the membrane phospholipid phosphatidylserine, procoagulant factors, and receptors. Coagulation is initiated on tissue factor-bearing cells (such as fibroblasts) with the formation of the extrinsic tenase complex TF:VIIa:Ca^{2+} , which activates factors IX and X and produces enough thrombin to activate platelets and factors V, VIII, and XI in a feedback loop. Coagulation is then propagated on the surface of the platelet with the formation of the intrinsic tenase complex ($\text{IXa:VIIIa:phospholipid:Ca}^{2+}$) and the prothrombinase complex ($\text{Xa:Va:phospholipid:Ca}^{2+}$), ultimately generating a burst of thrombin at the site of injury. See subsequent text for more details.

Erythrocytes, monocytes, and lymphocytes also participate in hemostasis. Erythrocytes add bulk and structural integrity to the fibrin clot; there is a tendency to bleed in anemia. In inflammatory conditions, monocytes and lymphocytes, and possibly ECs, provide surface-borne tissue factor that may trigger coagulation. Leukocytes also have a series of membrane integrins and selectins that bind adhesion molecules and help stimulate the production of inflammatory cytokines that promote the wound-healing process.²⁷

TABLE 37-4 Platelet Granule Contents

Platelet α -Granules	Platelet Dense Granules (Dense Bodies)
Large Molecules	Small Molecules
β -Thromboglobulin	Adenosine diphosphate (activates neighboring platelets)
Factor V	Adenosine triphosphate
Factor XI	Calcium
Protein S	Serotonin (vasoconstrictor)
Fibrinogen	
VWF	
Platelet factor 4 (heparin inhibitor)	
Platelet-derived growth factor	

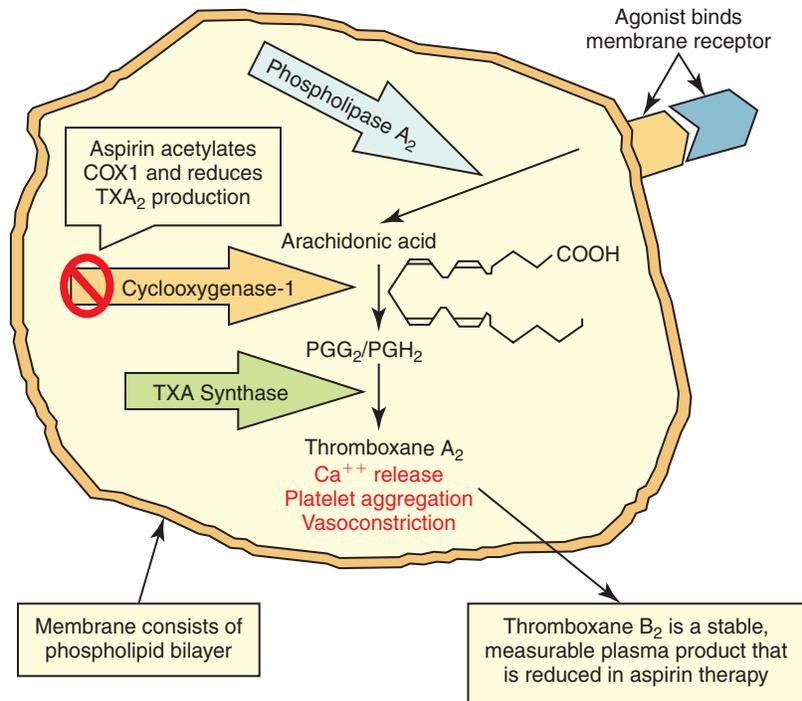


Figure 37-6 Arachidonic acid and aspirin effect. Phospholipase A₂ converts membrane phospholipids to arachidonic acid during platelet activation. Arachidonic acid is converted to prostaglandin endoperoxides (PGG₂/PGH₂) by cyclooxygenase, then to thromboxane A₂ (TXA₂). TXA₂ causes release of Ca²⁺, which promotes platelet aggregation and vasoconstriction. Aspirin permanently blocks the action of cyclooxygenase-1 and TXA₂ synthesis, impairing platelet function.

COAGULATION SYSTEM

Nomenclature of Procoagulants

Plasma transports at least 16 procoagulants, also called *coagulation factors*. Nearly all are glycoproteins synthesized in the liver, although monocytes, ECs, and megakaryocytes produce a few (Table 37-5; Figure 37-7). Eight are enzymes that circulate in an inactive form called *zymogens*. Others are *cofactors* that bind, stabilize, and enhance the activity of their respective enzymes. The sequence of activation is shown in Figure 37-8. During clotting, the procoagulants become activated and produce a localized thrombus. In addition, there are plasma glycoproteins that act as controls to regulate the coagulation process. See subsequent text for more details.

In 1958 the International Committee for the Standardization of the Nomenclature of the Blood Clotting Factors officially named the plasma procoagulants using Roman numerals in the order of their initial description or discovery.²⁸ When a procoagulant becomes activated, a lowercase *a* appears behind the numeral; for instance, activated factor VII is VIIa. Both zymogens and cofactors become activated in the coagulation process.

We customarily call factor I *fibrinogen* and factor II *prothrombin*, although occasionally they are identified by their numerals. The numeral III was given to *tissue thromboplastin*, a crude mixture of tissue factor and phospholipid. Now that the precise structure of tissue factor has been described, the numeral designation is seldom used. The numeral IV identified the plasma cation calcium (Ca²⁺); however, calcium is referred to by its name or chemical symbol, not by its numeral. The numeral VI was assigned to a procoagulant that later was determined to be activated factor V; VI was withdrawn from the

naming system and never reassigned. Factor VIII, antihemophilic factor, is a cofactor that circulates linked to a large carrier protein, VWF. Prekallikrein (pre-K), also called *Fletcher factor*, and high-molecular-weight kininogen (HMWK), also called *Fitzgerald factor*, have never received Roman numerals because they belong to the kallikrein and kinin systems, respectively, and their primary functions lie within these systems. Platelet phospholipids, particularly phosphatidylserine, are required for the coagulation process but were given no Roman numeral; instead they were once called collectively *platelet factor 3*.

Classification and Function of Procoagulants

The plasma procoagulants may be serine proteases or cofactors, except for factor XIII, which is a transglutaminase (Table 37-6).²⁹ Serine proteases are proteolytic enzymes of the trypsin family and include the procoagulants thrombin (factor IIa); factors VIIa, IXa, Xa, XIa, and XIIa; and pre-K.³⁰ Each member has a reactive seryl amino acid residue in its active site and acts on its substrate by hydrolyzing peptide bonds, digesting the primary backbone, and producing smaller polypeptide fragments. Serine proteases are synthesized as inactive zymogens consisting of a single peptide chain. Activation occurs when the zymogen is cleaved at one or more specific sites by the action of another protease during the coagulation process.

The procoagulant cofactors that participate in complex formation are tissue factor, located on membranes of fibroblasts and smooth muscle cells, and soluble plasma factors V, VIII, and HMWK. The remaining components of the coagulation pathway are fibrinogen, factor XIII, phospholipids, calcium, and VWF (Box 37-3). Fibrinogen is the ultimate substrate of the coagulation pathway. When hydrolyzed by thrombin,

TABLE 37-5 Plasma Procoagulants: Function, Molecular Weight, Plasma Half-Life, and Plasma Concentration

Factor	Name	Function	Molecular Weight (Daltons)	Half-Life (Hours)	Mean Plasma Concentration
I*	Fibrinogen	Thrombin substrate, polymerizes to form fibrin	340,000	100–150	200–400 mg/dL
II*	Prothrombin	Serine protease	71,600	60	10 mg/dL
III*	Tissue factor	Cofactor	44,000	Insoluble	None
IV*	Ionic calcium	Mineral	40	NA	8–10 mg/dL
V	Labile factor	Cofactor	330,000	24	1 mg/dL
VII	Stable factor	Serine protease	50,000	6	0.05 mg/dL
VIII	Antihemophilic factor	Cofactor	260,000	12	0.01 mg/dL
VWF	von Willebrand factor	Factor VIII carrier and platelet adhesion	600,000–20,000,000	24	1 mg/dL
IX	Christmas factor	Serine protease	57,000	24	0.3 mg/dL
X	Stuart-Prower factor	Serine protease	58,800	48–52	1 mg/dL
XI	Plasma thromboplastin antecedent (PTA)	Serine protease	143,000	48–84	0.5 mg/dL
XII	Hageman factor	Serine protease	84,000	48–70	3 mg/dL
Prekallikrein	Fletcher factor, pre-K	Serine protease	85,000	35	35–50 μ g/mL
High-molecular-weight kininogen	Fitzgerald factor, HMWK	Cofactor	120,000	156	5 mg/dL
XIII	Fibrin-stabilizing factor (FSF)	Transglutaminase, transamidase	320,000	150	2 mg/dL
Platelet factor 3	Phospholipids, phosphatidylserine, PF3	Assembly molecule	—	Released by platelets	—

*These factors are customarily identified by name rather than Roman numeral.

From Greenberg DL, Davie EW: The blood coagulation factors: their complementary DNAs, genes, and expression. In Colman RW, Marder VJ, Clowes, AM, et al, editors: *Hemostasis and thrombosis: basic principles and clinical practice*, ed 5, Philadelphia, 2006, Lippincott Williams & Wilkins, pp. 21–58.

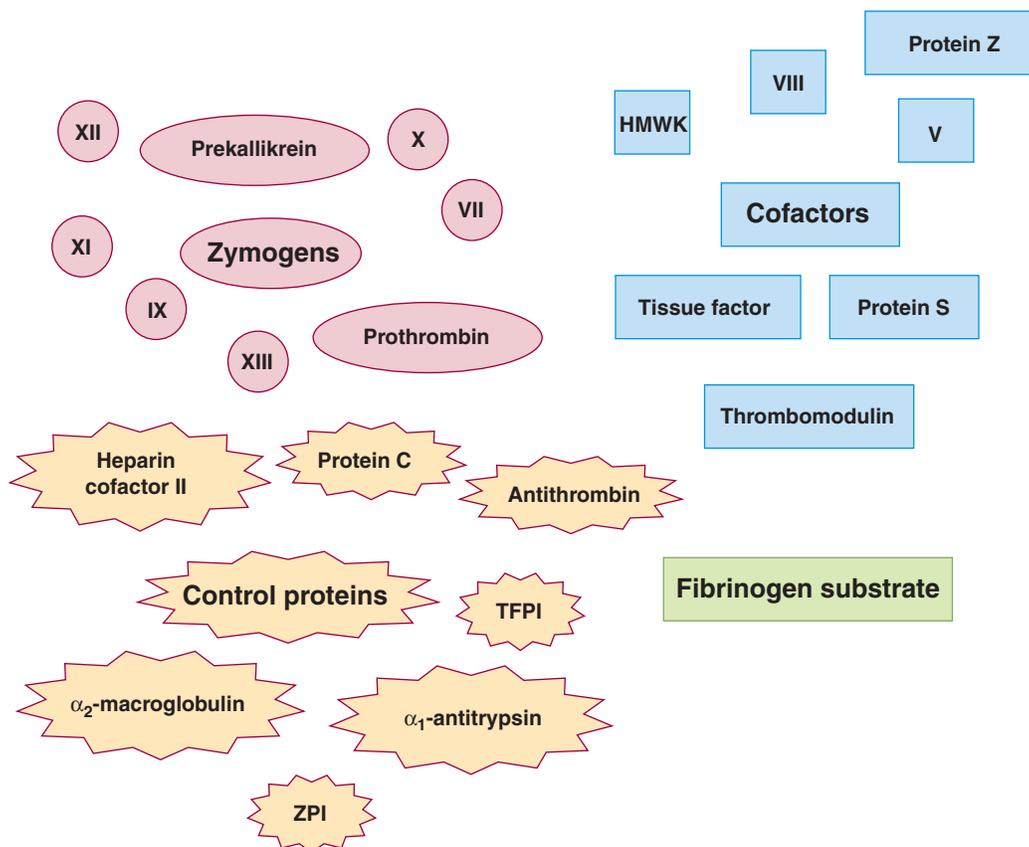


Figure 37-7 Procoagulants (zymogens), cofactors, and anticoagulants (control proteins). *HMWK*, High-molecular-weight kininogen; *TFPI*, tissue factor pathway inhibitor; *ZPI*, protein Z-dependent protease inhibitor.

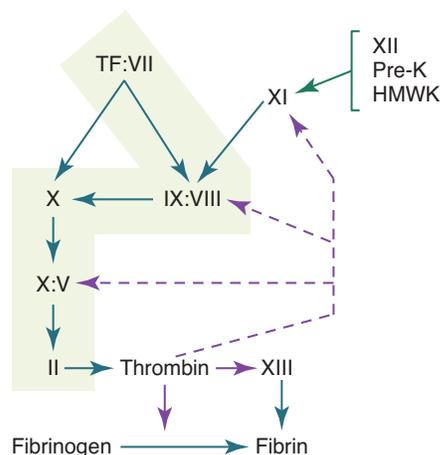


Figure 37-8 Simplified coagulation pathway. Exposed tissue factor (TF) activates factor VII, which activates factors IX and X. Factor IXa:VIIIa complex also activates X, and the factor Xa:Va complex activates prothrombin (factor II). The resulting thrombin cleaves fibrinogen to form fibrin and activates factor XIII to stabilize the clot. Thrombin also activates factors V, VIII, XI, and platelets. In vitro exposure to negatively charged surfaces activates the contact factors XII, pre-kallikrein (*pre-K*) and high-molecular-weight kininogen (*HMWK*), which activate factor XI.

TABLE 37-6 Plasma Procoagulant Serine Proteases

Inactive Zymogen	Active Protease	Cofactor	Substrate
Prothrombin (II)	Thrombin (IIa)	—	Fibrinogen, V, VIII, XI, XIII
VII	VIIa	Tissue factor	IX, X
IX	IXa	VIIIa	X
X	Xa	Va	Prothrombin
XI	XIa	—	IX
XII	XIIa	High-molecular-weight kininogen	XI
Prekallikrein	Kallikrein	High-molecular-weight kininogen	XI

BOX 37-3 Other Plasma Procoagulants

Fibrinogen
Factor XIII
Phospholipids
Calcium
VWF

fibrinogen forms the primary structural protein of the fibrin clot, which is further stabilized by factor XIII.³¹

Calcium is required for the assembly of coagulation complexes on platelet or cell membrane phospholipids. Serine proteases bind to negatively charged phospholipid surfaces, predominantly phosphatidylserine, through positively charged calcium ions. Activation is a localized cell-surface process, limited to the site of injury and controlled by regulatory mechanisms. If zymogen activation is uncontrolled and generalized, the condition is called *disseminated intravascular coagulation (DIC)*, a serious, often life-threatening condition (Chapter 39).

The molecular weights, plasma concentrations, and plasma half-lives of the procoagulants are given in Table 37-5. These essential pieces of clinical information assist in the interpretation of laboratory tests, monitoring of anticoagulant therapy, and design of effective replacement therapies in deficiency-related hemorrhagic diseases. For example, factor VIII has a short half-life of 12 hours, so replacement therapy for hemophilic individuals who are deficient in factor VIII is administered every 12 hours. For most factors, the level that achieves hemostatic effectiveness is 25% to 30%. This is the minimum level that must be maintained to prevent bleeding in factor-deficient patients. Therapy for a hemophilic patient is designed to maintain the factor level above 30%. A higher level may be desirable, such as in a patient preparing for surgery. The half-life is also important in monitoring anticoagulant therapy, especially warfarin (Coumadin), because even though factor VII becomes reduced in 6 hours, the reduction of prothrombin takes 4 to 5 days. Therefore, the full effect of warfarin is not realized until approximately 5 days after therapy has begun.

Vitamin K-Dependent Prothrombin Group

Prothrombin (factor II), factors VII, IX, and X and the regulatory proteins protein C, protein S, and protein Z are vitamin K-dependent (Table 37-7). These are named the *prothrombin group* because of their structural resemblance to prothrombin. All seven proteins have 10 to 12 glutamic acid units near their amino termini. All except proteins S and Z are serine proteases when activated; S and Z are cofactors.

Vitamin K is a quinone found in green leafy vegetables (Box 37-4) and is produced by the intestinal organisms *Bacteroides*

TABLE 37-7 Vitamin K-Dependent Coagulation Factors

Procoagulants	Regulatory Proteins
Prothrombin (II)	Protein C
VII	Protein S
IX	Protein Z
X	

BOX 37-4 Food Sources High in Vitamin K

Kale
Spinach
Turnip greens
Collards
Mustard greens
Swiss chard
Brussels sprouts
Broccoli
Asparagus
Cabbage
Green onions
Lettuce: Boston, romaine, or Bibb
Avocado
Cauliflower
Parsley, fresh

fragilis and *Escherichia coli*. Vitamin K catalyzes an essential post-translational modification of the prothrombin group proteins: γ -carboxylation of amino-terminal glutamic acids (Figure 37-9). Glutamic acid is modified to γ -carboxyglutamic acid when a second carboxyl group is added to the γ carbon. With two ionized carboxyl groups, the γ -carboxyglutamic acids gain a net negative charge, which enables them to bind ionic calcium (Ca^{2+}). The bound calcium enables the vitamin K-dependent proteins to bind to negatively charged phospholipids to form coagulation complexes.

In vitamin K deficiency or in the presence of warfarin, a vitamin K antagonist, the vitamin K-dependent procoagulants are released from the liver without the second carboxyl group

added to the γ carbon. These are called *des*- γ -carboxyl proteins or *proteins in vitamin K antagonism* (PIVKAs). Because they lack the second carboxyl group, they cannot bind to Ca^{2+} and phospholipid, so they cannot participate in the coagulation reaction. Vitamin K antagonism is the basis for oral anticoagulant (warfarin, Coumadin) therapy (Chapter 43).

Vitamin K-dependent procoagulants are essential for the assembly of three membrane complexes leading to the generation of thrombin (Figure 37-10). Each complex is composed of a vitamin K-dependent serine protease, its non-enzyme cofactor, and Ca^{2+} , bound to the negatively charged phospholipid membranes of activated platelets or tissue factor-bearing cells. The initial complex, extrinsic tenase, is composed of factor

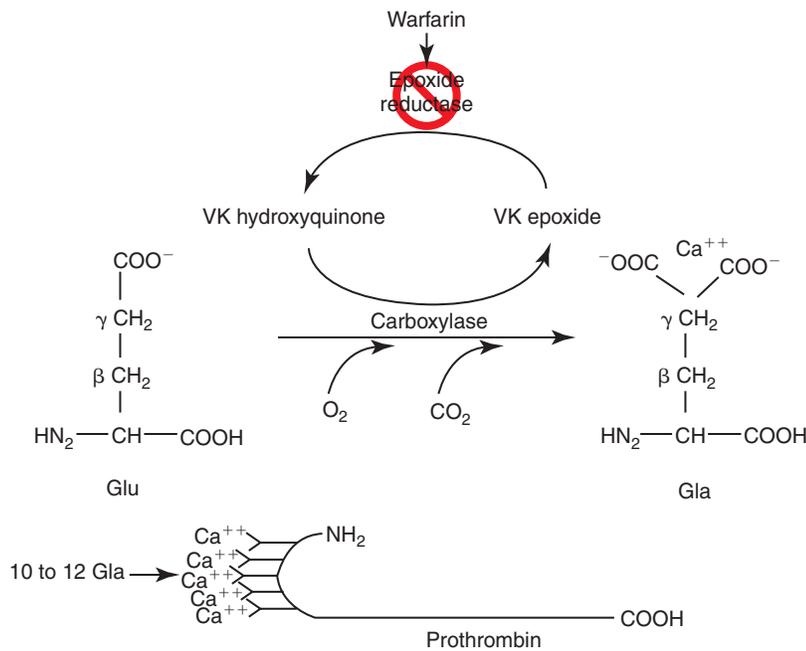


Figure 37-9 Vitamin K (*K*) posttranslational γ -carboxylation of coagulation factors II (prothrombin), VII, IX, and X, and control proteins C, S, and Z. Vitamin K hydroxyquinone transfers a carboxyl (COO^-) group to the γ carbon of glutamic acid (*Glu*), creating γ -carboxyglutamic acid (*Gla*). The negatively charged pocket formed by the two carboxyl groups attracts ionic calcium, which enables the molecule to bind to phosphatidylserine. Vitamin K hydroxyquinone is oxidized to vitamin K epoxide by *carboxylase* in the process of transferring the carboxyl group but is subsequently reduced to the hydroxyquinone form by *epoxide reductase*. Warfarin suppresses *epoxide reductase*, which slows the reaction and prevents γ -carboxylation. “Des-carboxy” proteins are unable to participate in coagulation. There are typically 10 to 12 γ -carboxyglutamic acid molecules near the amino terminus of the vitamin K-dependent factors.

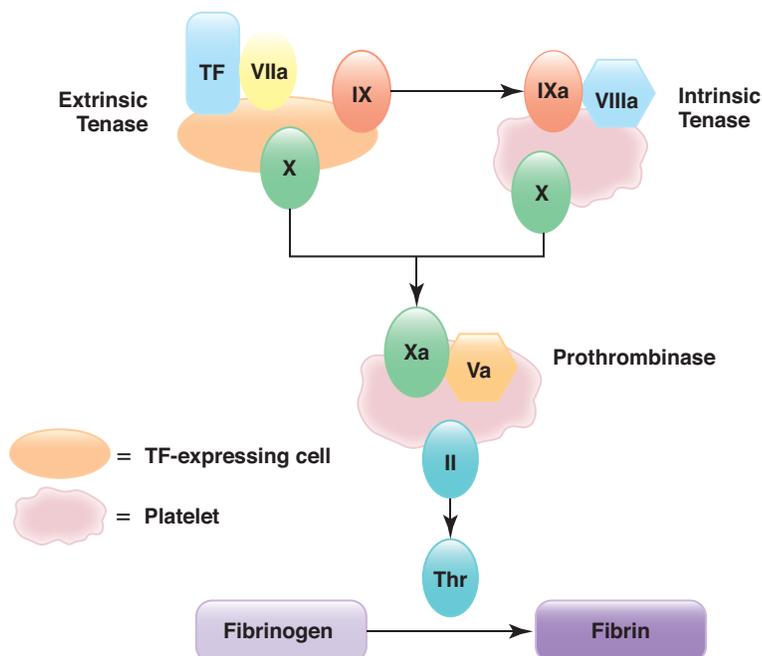


Figure 37-10 Coagulation complexes. Coagulation complexes form on TF-bearing cells (TF:VIIa) and on platelet phospholipid membranes (IXa:VIIIa and Xa:Va). Each complex consists of a vitamin K-dependent serine protease, a cofactor, and Ca^{2+} , bound to the cell membrane. *Extrinsic tenase complex* is factor VIIa and tissue factor (TF) on the membrane of a TF-bearing cell. This complex activates both factors IX and X. *Intrinsic tenase complex*, which is factor IXa and its cofactor VIIIa on platelet membranes, activates factor X also. *Prothrombinase complex* is factor Xa and its cofactor Va, bound to the surface of platelets. Prothrombinase cleaves prothrombin to the active enzyme, thrombin.

TABLE 37-8 Coagulation Complexes

Complex	Components	Activates
Extrinsic tenase	VIIa, tissue factor, phospholipid, and Ca ²⁺	IX and X
Intrinsic tenase	IXa, VIIIa, phospholipid, and Ca ²⁺	X
Prothrombinase	Xa, Va, phospholipid, and Ca ²⁺	Prothrombin

VIIa and tissue factor, and it activates factors IX and X, which are components of the next two complexes, intrinsic tenase and prothrombinase, respectively (Table 37-8). Intrinsic tenase is composed of factor IXa and its cofactor VIIIa; it also activates factor X much more efficiently than the TF:VIIa complex. Prothrombinase is composed of factor Xa and its cofactor Va; this converts prothrombin to thrombin in a multistep hydrolytic process that releases thrombin and a peptide fragment called prothrombin fragment 1.2 (F 1.2). Prothrombin fragment 1.2 in plasma is thus a marker for thrombin generation.

Cofactors in Hemostasis

Procoagulant cofactors are tissue factor, factor V, factor VIII, and HMWK. Coagulation control cofactors are thrombomodulin, protein S, and protein Z (Table 37-9).³² Thrombomodulin is also a cofactor in control of fibrinolysis. Each cofactor binds its particular serine protease. When bound to their cofactors, serine proteases gain stability and increased reactivity.

Tissue factor is a transmembrane receptor for factor VIIa and is found on extravascular cells such as fibroblasts and smooth muscle cells, but under normal conditions, it is not found on blood vessel ECs.³³ Vessel injury exposes blood to the subendothelial tissue factor-bearing cells and leads to activation of coagulation through VIIa. Tissue factor is expressed in high levels in cells of the brain, lung, placenta, heart, kidney, and testes. In inflammatory conditions and sepsis, leukocytes and other cells can also express tissue factor and initiate coagulation.³⁴

Factors V and VIII are soluble plasma proteins. Both are activated by thrombin and inactivated by protein C. Factor V is a glycoprotein circulating in plasma and also present in platelet α -granules. During platelet activation and secretion, platelets release partially activated factor V at the site of injury. Factor Va is a cofactor to Xa in the prothrombinase complex in

TABLE 37-9 Hemostasis Cofactors

Cofactor	Function	Binds
Tissue factor	Procoagulant	VIIa
V	Procoagulant	Xa
VIII	Procoagulant	IXa
High-molecular-weight kininogen	Procoagulant	XIIa, prekallikrein
Thrombomodulin	Control (Protein C) Antifibrinolytic (TAFI)	Thrombin Thrombin
Protein S	Control	Protein C, TFPI
Protein Z	Control	ZPI

coagulation. The prothrombinase complex accelerates thrombin generation more than 300,000-fold compared to Xa alone.³⁵ As described below, thrombomodulin-bound thrombin activates protein C, which inactivates Va to Vi. Therefore, factor V is both activated and then ultimately inactivated by the generation of thrombin, as is factor VIII. Factor VIII is a cofactor to factor IX, which together form the intrinsic tenase complex, discussed in the next section. High-molecular-weight kininogen is a cofactor to factor XIIa and prekallikrein in the intrinsic contact factor complex, a mechanism for activating coagulation in conditions where foreign objects such as mechanical heart valves or bacterial membranes and/or high levels of inflammation are present.

Thrombomodulin, a transmembrane protein constitutively expressed by vascular ECs, is a thrombin cofactor. Together, thrombomodulin and thrombin activate protein C, a coagulation regulatory protein, and thrombin activatable fibrinolysis inhibitor (TAFI), a fibrinolysis inhibitor. In one of many examples of negative feedback regulation in coagulation, once thrombin is bound to thrombomodulin, it loses its procoagulant ability to activate factors V and VIII, and, through activation of protein C, leads to destruction of factors V and VIII, thus suppressing further generation of thrombin.

Both protein S and protein C are cofactors in the regulation and control of coagulation, discussed later in this chapter. Protein S is a cofactor to protein C, as well as TFPI. Protein Z is a cofactor to Z-dependent protease inhibitor (ZPI).

Factor VIII and Von Willebrand Factor

Factor VIII has a molecular mass of 260,000 Daltons and is produced primarily by hepatocytes, but also by microvascular ECs in lung and other tissues.³⁶ Free factor VIII is unstable in plasma; it circulates bound to VWF. During coagulation, thrombin cleaves factor VIII from VWF and activates it. Factor VIIIa binds to activated platelets and forms the intrinsic tenase complex with factor IXa and Ca²⁺. Like factor Va, factor VIIIa is also inactivated by protein C.

Factor VIII and factor IX are the two plasma procoagulants whose production is governed by genes carried on the X chromosome. Hemophilia A (factor VIII deficiency) and hemophilia B (factor IX deficiency) are therefore sex-linked disorders occurring almost exclusively in males. Males with hemophilia A have diminished factor VIII activity but normal VWF levels.³⁷ Factor VIII is a cofactor, but its importance in hemostasis cannot be overstated, as evidenced by the severe bleeding and symptoms associated with hemophilia A.

Factor VIII deteriorates more rapidly than the other coagulation factors in stored blood. In thawed component plasma, the factor VIII level drops to approximately 50% after 5 days.³⁸ Treatment for hemophilia bleeding episodes consists of replacement therapy transfused according to the 12-hour half-life of factor VIII.

VWF is a large multimeric glycoprotein that participates in platelet adhesion and transports the procoagulant factor VIII. VWF is composed of multiple subunits of 240,000 Daltons each.³⁹ The subunits are produced by ECs and megakaryocytes, where they combine to form multimers that range from 600,000 to 20,000,000 Daltons.⁴⁰ VWF molecules are stored in

α -granules in platelets and in Weibel-Palade bodies in ECs. The molecules are released from storage into the plasma, and they circulate at a concentration of 7 to 10 $\mu\text{g/mL}$. ECs release ultra-large multimers of VWF into plasma, where they are normally degraded into smaller multimers by a VWF-cleaving protease, ADAMTS-13 (*a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13*), in blood vessels with high shear stress. In thrombotic thrombocytopenic purpura (TTP), inherited or acquired defective ADAMTS-13 enzyme activity is associated with the presence of ultralarge VWF multimers in plasma, resulting in platelet aggregation and microvascular thrombosis.⁴¹

VWF has receptor sites for both platelets and collagen (Figure 37-11) and helps to bind platelets to exposed subendothelial collagen during platelet adhesion, especially in arteries and arterioles where the flow of blood is faster. The primary platelet surface receptor for VWF is GP Ib/IX/V. Arginine-glycine-aspartic acid (RGD) sequences in VWF also bind a second platelet integrin, GP IIb/IIIa, during platelet aggregation.

A third site on the VWF molecule binds collagen, and a fourth site binds the plasma procoagulant cofactor, factor VIII. VWF is decreased in von Willebrand disease (VWD), a relatively common disorder that occurs in 1% to 2% of the general population. Because factor VIII depends on VWF for stability, individuals with VWD who have diminished VWF also have diminished factor VIII activity levels. Typically, factor VIII levels decrease to hemorrhagic levels (less than 30%) only in severe VWD. The level of VWF also varies in people according to their ABO blood type. Group O individuals have lower levels of VWF than other ABO types.⁴² VWF is an acute phase protein, as is factor VIII, and levels increase in pregnancy, trauma, infections, and stress.

Factor XI and the Contact Factors

The “contact factors,” also called intrinsic accessory pathway proteins, are factor XII, high-molecular-weight kininogen (HMWK, Fitzgerald factor), and prekallikrein (pre-K, Fletcher factor). They are so named because they are activated by contact with negatively charged foreign surfaces. Factor XIIa transforms pre-K, a glycoprotein that circulates bound to HMWK, into its active form kallikrein, which cleaves HMWK to form bradykinin.

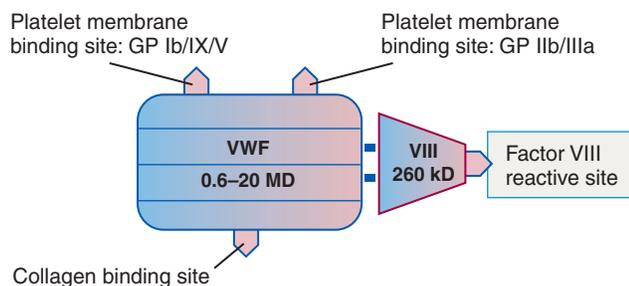


Figure 37-11 Von Willebrand factor (VWF)–factor VIII complex. Factor VIII circulates covalently bound to VWF. VWF provides three other active receptor sites: VWF binds to collagen and binds to glycoprotein Ib/IX/V to support platelet adhesion and binds to glycoprotein IIb/IIIa to facilitate platelet aggregation.

Factor XII and pre-K are zymogens that are activated to become serine proteases; HMWK is a nonenzymatic cofactor.

The contact factor complex (HMWK:pre-K:FXII) activates factor XI; factor XIa is an activator of factor IX (Figure 37-8). Deficiencies of factor XII, HMWK, or pre-K do not cause clinical bleeding disorders. However, deficiencies do prolong laboratory tests and necessitate investigation. Factor XII is activated in vitro by negatively charged surfaces such as nonsiliconized glass, kaolin, or ellagic acid in partial thromboplastin time (PTT) reagents. In vivo, foreign materials such as stents or valve prostheses may activate contact factors to cause thrombosis.

Factor XI is activated by the contact factor complex and, more significantly, by thrombin during coagulation generated from tissue factor activation. Factor XIa activates factor IX, and the reaction proceeds as described previously. Deficiencies of factor XI (Rosenthal syndrome) usually result in mild and variable bleeding.⁴³ Factor XI supplements or boosts factor IX activation, so deficiencies of factor XI are less severe clinically than deficiencies of the other factors such as IX or VIII.

Thrombin

The primary function of thrombin is to cleave fibrinopeptides A and B from the α and β chains of the fibrinogen molecule, triggering spontaneous fibrin polymerization (Figure 37-12). In addition, thrombin amplifies the coagulation mechanism by activating cofactors V and VIII and factor XI by a positive feedback mechanism (Figure 37-8). Thrombin also activates factor XIII, which forms covalent bonds between the D domains of the fibrin polymer to cross-link and stabilize the fibrin clot. Thrombin also initiates aggregation of platelets. Thrombin bound to thrombomodulin activates the protein C pathway to suppress coagulation, and it activates TAFI to suppress fibrinolysis. Thrombin, therefore, plays a role in coagulation (fibrin), in platelet activation, in coagulation control (protein C), and in controlling fibrinolysis (TAFI). Because of its multiple autocatalytic functions, thrombin is considered the key protease of the coagulation pathway.

Fibrinogen Structure and Fibrin Formation, Factor XIII

Fibrinogen is the primary substrate of thrombin, which converts soluble fibrinogen to insoluble fibrin to produce a clot. Fibrinogen is also essential for platelet aggregation because it links activated platelets through their GP IIb/IIIa platelet fibrinogen receptor. Fibrinogen is a 340,000 Dalton glycoprotein synthesized in the liver. The normal plasma concentration of fibrinogen ranges from 200 to 400 mg/dL, the most concentrated of all the plasma procoagulants. Fibrinogen is an acute phase reactant protein, whose level increases in inflammation, infection, and other stress conditions. Platelet α -granules absorb, transport, and release abundant fibrinogen.⁴⁴

The fibrinogen molecule is a mirror-image “trinodular” dimer, each half consisting of three nonidentical polypeptides, designated $\text{A}\alpha$, $\text{B}\beta$, and γ , united by disulfide bonds (Figure 37-13). The six N-terminals assemble to form a bulky central region called the E domain. The carboxyl terminals assemble at the outer ends of the molecule to form two D domains.⁴⁵

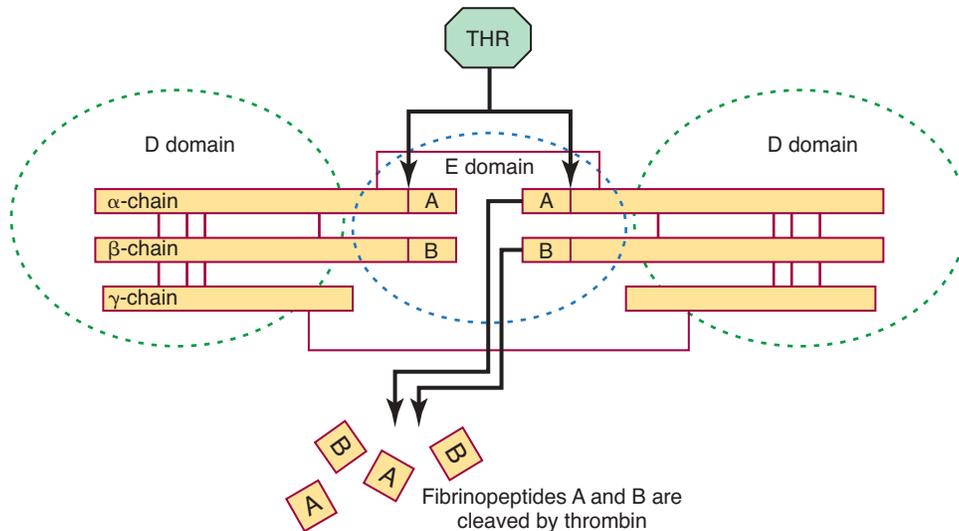


Figure 37-12 Fibrinogen domains and cleavage by thrombin. Each molecule of fibrinogen or fibrin has three “domains”: two D domains (the carboxyl ends of the molecule) and one E domain (the central portion of the molecule), as shown in this diagram. Thrombin (*THR*) cleaves fibrinopeptides A and B from the alpha and beta chains in the E domain.

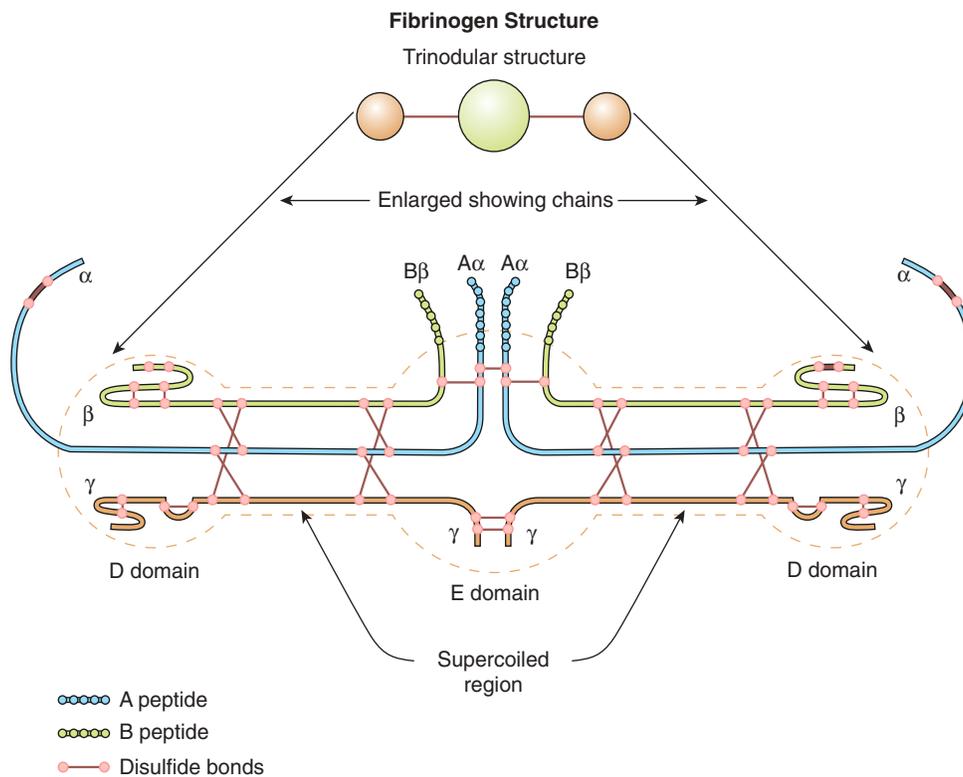


Figure 37-13 Structure of fibrinogen. Fibrinogen is a trinodular structure composed of three pairs ($A\alpha$, $B\beta$, and γ) of disulfide-bonded polypeptide chains. The central node is known as the *E domain*. Thrombin cleaves small peptides, A and B, from the α and β chains in this region to form fibrin. The central nodule is joined by supercoiled α -helices to the terminal nodules known as the *D domains*. (From McKenzie SB, Williams JL: *Clinical laboratory hematology*, ed 2, Upper Saddle River, NJ, 2009, Pearson, p 653.)

Thrombin cleaves fibrinopeptides A and B from the protruding N-termini of each of the two α and β chains of fibrinogen, reducing the overall molecular weight by 10,000 Daltons. The cleaved fibrinogen is called *fibrin monomer*. The exposed fibrin monomer α and β chain ends (E domain) have an immediate affinity for portions of the D domain of neighboring

monomers, spontaneously polymerizing to form *fibrin polymer* (Figure 37-14).

Thrombin also activates factor XIII, a heterodimer whose α subunit is produced mostly by megakaryocytes and monocytes, and whose β subunit is produced in the liver.⁴⁶ Factor XIIIa covalently crosslinks fibrin polymers to form a stable

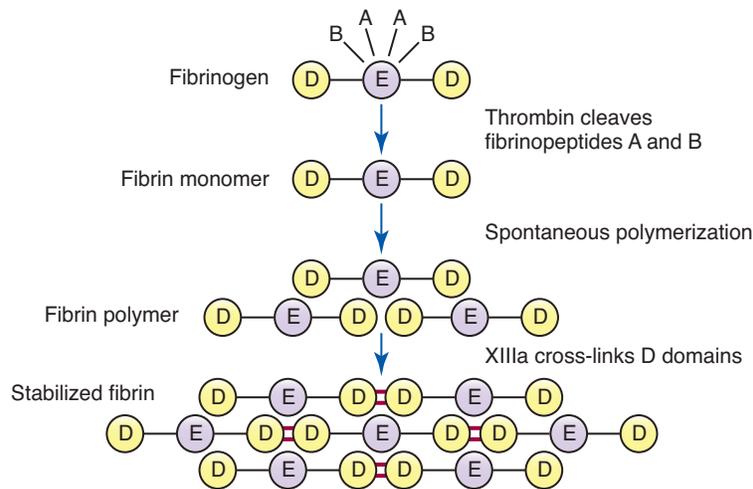


Figure 37-14 Formation of a stabilized fibrin clot. Thrombin cleaves fibrinopeptides A and B to form fibrin monomer. Fibrin monomers polymerize due to the affinity of thrombin-cleaved positively charged E domains for negatively charged D domains of other monomers. Factor XIIIa catalyzes the covalent cross-linking of γ chains of adjacent D domains to form an insoluble stable fibrin clot.

insoluble fibrin clot. Factor XIIIa is a transglutaminase that catalyzes the formation of covalent bonds between the carboxyl terminals of γ chains from adjacent D domains in the fibrin polymer. These bonds link the ϵ -amino acid of lysine moieties and the γ -amide group of glutamine units. Multiple cross-links form to provide an insoluble meshwork of fibrin polymers linked by their D domains, providing physical strength to the fibrin clot. Factor XIIIa reacts with other plasma and cellular structural proteins and is essential to wound healing and tissue integrity. Cross-linking of fibrin polymers by factor XIIIa covalently incorporates fibronectin, a plasma protein involved in cell adhesion, and α_2 -antiplasmin, rendering the fibrin mesh resistant to fibrinolysis. Plasminogen, the primary serine protease of the fibrinolytic system, also becomes covalently bound via lysine moieties, as does TPA, a serine protease that ultimately hydrolyzes and activates bound plasminogen to initiate fibrinolysis.

Plasma-Based (In Vitro) Coagulation: Extrinsic, Intrinsic, and Common Pathways

In the past, two coagulation pathways were described, both of which activated factor X at the start of a common pathway leading to thrombin generation (Figure 37-15). The pathways were characterized as cascades in that as one enzyme became activated, it in turn activated the next enzyme in sequence. Most coagulation experts identified the activation of factor XII as the primary step in coagulation because this factor could be found in blood, whereas tissue factor could not. Consequently, the reaction system that begins with factor XII and culminates in fibrin polymerization has been called the *intrinsic pathway*. The coagulation factors of the intrinsic pathway, in order of reaction, are XII, pre-K, HMWK, XI, IX, VIII, X, V, prothrombin (II), and fibrinogen. The laboratory test that detects the absence of one or more of these factors is the activated partial thromboplastin time (APTT or PTT; Chapter 42). We now know that the contact factors XII, pre-K, and HMWK do not play a significant role in *in vivo* coagulation with trauma-type

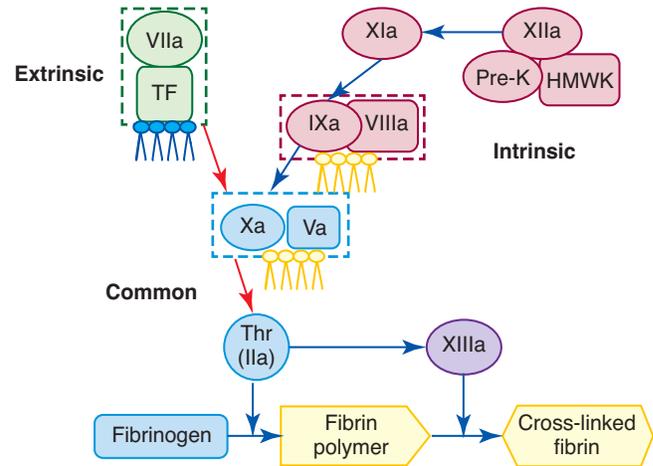


Figure 37-15 Plasma-based *in vitro* coagulation: intrinsic, extrinsic, and common pathways. In the intrinsic pathway, the contact factors XII, prekallikrein (*pre-K*), and high-molecular-weight kininogen (*HMWK*) are activated and proceed to activate factors XI, IX, VIII, X, and V and prothrombin, which converts fibrinogen to fibrin. In the extrinsic pathway, exposed tissue factor (*TF*) on subendothelial cells activates factor VII, which activates factors X, V, and prothrombin, cleaving fibrinogen to fibrin. Both the intrinsic and extrinsic pathways converge with the activation of factor X, so factors X, V, prothrombin, and fibrinogen are called the common pathway.

injuries, although their deficiencies prolong the *in vitro* laboratory tests of the intrinsic pathway, in particular, the PTT.

Formation of TF:VIIa has since proven to be the primary *in vivo* initiation mechanism for coagulation. Because tissue factor is not present in blood, the tissue factor pathway has been called the *extrinsic pathway*. This pathway includes the factors VII, X, V, prothrombin, and fibrinogen. The test used to measure the integrity of the extrinsic pathway is the prothrombin time test (PT; Chapter 42).

The PT and PTT are assays often used in tandem to screen for coagulation factor deficiencies. Factor VIII and factor IX are not considered to be part of the extrinsic pathway, because the PT fails to identify their absence or deficiency. But clearly,

the IXa:VIIIa complex in the intrinsic pathway is crucial to the activation of factor X. Deficiencies of either one of these components—factor VIII in hemophilia A, or factor IX in hemophilia B—can result in severe and life-threatening hemorrhage.

The two pathways have in common factor X, factor V, prothrombin, and fibrinogen; this portion of the coagulation pathway is often called the *common pathway*. These designations—*intrinsic*, *extrinsic*, and *common*—are used extensively to interpret *in vitro* laboratory testing and to identify factor deficiencies; however, they do not adequately describe the complex interdependent reactions that occur *in vivo*.

Cell-Based (In Vivo, Physiologic) Coagulation

An intricate combination of cellular and biochemical events function in harmony to keep blood liquid within the veins and arteries, to prevent blood loss from injuries by the formation of thrombi, and to reestablish blood flow during the healing process.⁴⁷ As noted above, the series of cascading proteolytic reactions traditionally known as the *extrinsic* and *intrinsic coagulation pathways* do not fully describe how coagulation occurs *in vivo*. These pathways are not distinct, independent, alternative mechanisms for generating thrombin but are actually interdependent. For example, a deficiency of factor VII in the extrinsic pathway can cause significant bleeding, even when the intrinsic pathway is intact. Similarly, deficiencies of factors VIII and IX may cause severe bleeding, regardless of the presence of a normal extrinsic pathway.⁴⁸

In addition to procoagulant and anticoagulant plasma proteins, normal physiologic coagulation requires the presence of two cell types for formation of coagulation complexes: cells that express tissue factor (usually extravascular) and platelets (intravascular) (Figure 37-16).⁴⁹ Operationally, coagulation can be described as occurring in two phases: initiation, which occurs on tissue factor-expressing cells and produces 3% to 5% of the total thrombin generated, and propagation,

occurring on platelets, which produces 95% or more of the total thrombin.⁵⁰

Initiation

In vivo, the principle mechanism for generating thrombin is begun by formation of the extrinsic tenase complex, rather than the intrinsic pathway. The initiation phase refers to extrinsic tenase complex formation and generation of small amounts of factor Xa, factor IXa, and thrombin (Figure 37-16).

Damage to the endothelium spills blood and platelets into the extravascular tissue and triggers a localized response. The magnitude of the response depends largely on the extent of the injury: how large the bleed is, how much tissue is damaged, and how many platelets are available.

About 1% to 2% of factor VIIa is present normally in blood in the activated form, but it is inert until bound to tissue factor⁵¹ and is unaffected by TFPI and other inhibitors. Fibroblasts and other subendothelial cells provide tissue factor, a cofactor to factor VIIa. Factor VIIa binds to tissue factor on the membrane of subendothelial cells, and the extrinsic tenase complex TF:VIIa is formed.

TF:VIIa activates low levels of both factor IX and factor X. Minute amounts of thrombin are generated by membrane-bound Xa and Xa:Va prothrombinase complexes. Factor Va comes from the activation of plasma factor V by thrombin, by platelets if there has been an injury, or by noncoagulation proteases.⁵²

Coagulation complexes bound to cell membranes are relatively protected from inactivation by most inhibitors. However, if Xa:Va dissociates from the cell, it is rapidly inactivated by the protease inhibitors TFPI, antithrombin, and protein Z–dependent protease inhibitor (ZPI) until a threshold of Xa:Va activity is reached.

Even though the amount of thrombin generated in this phase is minute, platelets, cofactors, and procoagulants become activated; fibrin formation begins; and the initial platelet

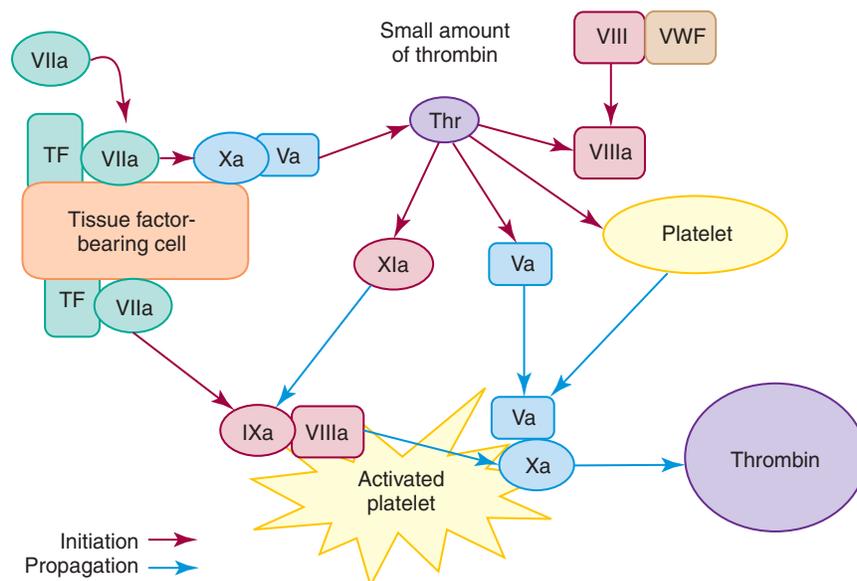


Figure 37-16 Cell-based *in vivo* physiologic coagulation. VIIa binds to tissue factor (TF) and activates both factors X and IX. Cell-bound factor Xa combines with Va and generates a small amount of thrombin (Thr), which activates platelets, V, VIII, and XI and begins fibrin formation. Factor IXa, activated by both TF:VIIa and XIa, combines with factor VIIIa on the platelet surface to activate X, which forms prothrombinase (Xa:Va) and produces a burst of thrombin.

plug is formed. The low level of thrombin generated in the initiation phase (1) activates platelets through cleavage of protease activated receptors PAR-1 and PAR-4; (2) activates factor V released from platelet α -granules; (3) activates factor VIII and dissociates it from VWF; (4) activates factor XI, the intrinsic accessory procoagulant that activates more factor IX; and (5) splits fibrinogen peptides A and B from fibrinogen and forms a preliminary fibrin network.

Cleavage of fibrinopeptides occurs at the end of the initiation phase and beginning of the propagation phase. In most clot-based coagulation assays, this is the visual endpoint of the assay.⁴⁵ It occurs with only 10 to 30 nmol/L of thrombin, or approximately 3% of the total thrombin generated.

Propagation

More than 95% of thrombin generation occurs during propagation. In this phase the reactions occur on the surface of the activated platelet, which now has all the components needed for coagulation. Large numbers of platelets adhere to the site of injury, localizing the coagulation response. Platelets are activated at the site of injury by both the low-level thrombin generated in the initiation phase and by adhering to exposed collagen (Figure 37-16). They are sometimes referred to as COAT-platelets: platelets partially activated by collagen and thrombin.⁵³

These partially activated COAT-platelets have a higher level of procoagulant activity than platelets exposed to collagen alone. They also provide a surface for formation and amplification of intrinsic tenase and prothrombinase complexes.

The cofactors Va and VIIIa activated by thrombin in the initiation phase bind to platelet membranes and become receptors for Xa and IXa. IXa generated in the initiation phase binds to VIIIa on the platelet membrane to form the intrinsic tenase complex IXa:VIIIa. More factor IXa is also generated by platelet-bound factor XIa. This intrinsic tenase complex activates factor X at a 50- to 100-fold higher rate than the extrinsic tenase complex.⁴⁹ Factor Xa binds to Va to form the prothrombinase complex, which activates prothrombin and generates a burst of thrombin. Thrombin cleaves fibrinogen into a fibrin clot, activates factor XIII to stabilize the clot, binds to thrombomodulin to activate the protein C control pathway, and activates TAFI to inhibit fibrinolysis.

Since coagulation depends on the presence of both tissue factor-bearing cells and activated platelets, clotting is localized to the site of injury. Protease inhibitors and intact endothelium prevent clotting from spreading to other parts of the body.

It may be helpful operationally to think of the extrinsic or tissue factor pathway as occurring on the tissue factor-bearing cell and the intrinsic pathway (minus factors XII, HMWK, and pre-K) as occurring on the platelet surface. However, these are not separate and redundant pathways; they are interdependent and occur in parallel until blood flow has ceased and termination by control mechanisms takes place.

Both platelets and tissue factor-bearing cells are essential for physiologic coagulation. Deficiencies of any of the key proteins of coagulation complex formation and activity (VII, IX, VIII, X, V, or prothrombin) compromise thrombin generation and manifest as significant bleeding disorders.

COAGULATION REGULATORY MECHANISMS

Inhibitors and their cofactors regulate serine proteases and cofactors in the coagulation system. They also provide feedback loops to maintain a complex and delicate balance between thrombosis and abnormal bleeding. These inhibitors, or natural anticoagulants, function to slow the activation of procoagulants and suppress thrombin production. They ensure that coagulation is localized and is not a systemic response, and they prevent excessive clotting or thrombosis. The principal regulators are TFPI, antithrombin (AT), and activated protein C, the endpoint of the protein C pathway. Acquired or inherited deficiencies of these proteins may be associated with increased incidence of venous thromboembolic disease, as the hemostatic balance is shifted more toward coagulation than termination of the activated pathway. Figure 37-17 illustrates coagulation mechanism regulatory points. Characteristics of these and other coagulation regulatory proteins are summarized in Table 37-10.

Tissue Factor Pathway Inhibitor

TFPI is a Kunitz-type serine protease inhibitor and is the principal regulator of the tissue factor pathway. The Kunitz-2 domain binds to and inhibits factor Xa, and Kunitz-1 binds to and inhibits VIIa:TF.⁵⁴ TFPI is synthesized primarily by ECs and is also expressed on platelets. In the initiation of coagulation, factor VIIa and tissue factor combine to activate factors IX and X. TFPI inhibits coagulation in a two-step process by first binding and inactivating Xa. The TFPI:Xa complex then binds to TF:VIIa, forming a quaternary complex and preventing further activation of X and IX (Figure 37-18).^{55,56} Alternatively, TFPI may bind to Xa in the TF:VIIa:Xa complex and inactivate Xa and TF:VIIa. TFPI provides feedback inhibition, because it is not actively engaged until coagulation is initiated and factor X is activated. Protein S, the cofactor of activated protein C

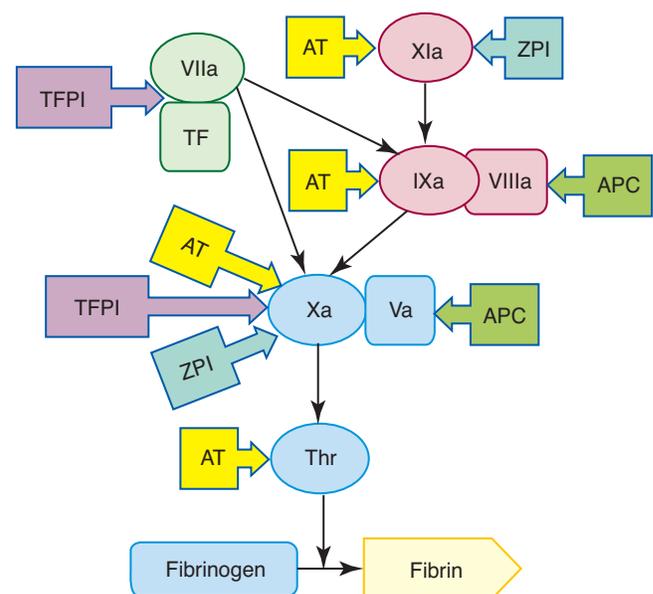


Figure 37-17 Coagulation pathway showing regulatory points. TFPI, Tissue factor pathway inhibitor; AT, antithrombin; APC, activated protein C; ZPI, protein Z-dependent protease inhibitor.

TABLE 37-10 Coagulation Regulatory Proteins

Name	Function	Molecular Mass (Daltons)	Half-Life (Hours)	Mean Plasma Concentration
Tissue factor pathway inhibitor	With Xa, binds TF:VIIa	33,000	Unknown	60–80 ng/mL
Thrombomodulin	EC surface receptor for thrombin	450,000	Does not circulate	None
Protein C	Serine protease	62,000	7–9	2–6 μg/mL
Protein S	Cofactor	75,000	Unknown	20–25 μg/mL
Antithrombin	Serpin	58,000	68	24–40 mg/dL
Heparin cofactor II	Serpin	65,000	60	30–70 μg/mL
Z-dependent protease inhibitor	Serpin	72,000	Unknown	1.5 μg/mL
α ₁ -Protease inhibitor (α ₁ -antitrypsin)	Serpin	60,000	Unknown	250 mg/dL
α ₂ -Macroglobulin	Serpin	725,000	60	150–400 mg/dL

Serpin, Serine protease inhibitor; *EC*, endothelial cell.

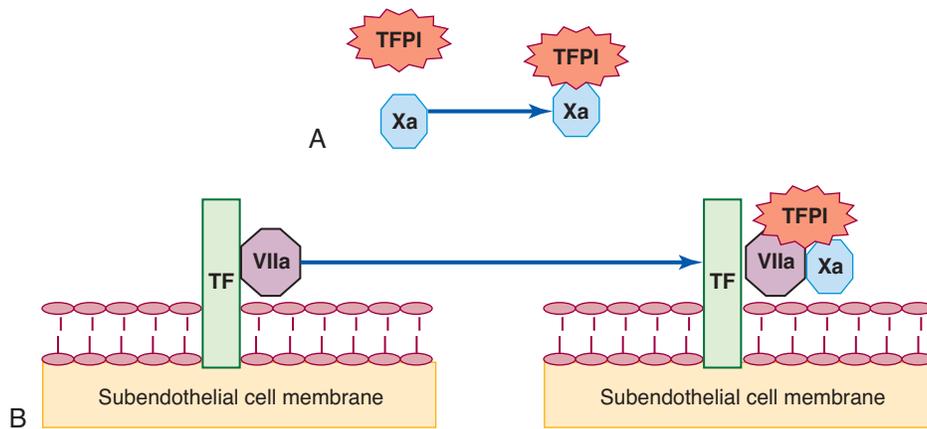


Figure 37-18 Tissue factor pathway inhibitor. *TFPI* binds the complex of tissue factor (*TF*) and factors *VIIa* and *Xa* in a *Xa*-dependent feedback mechanism. **A**, *TFPI* first binds to factor *Xa* and inactivates it. **B**, The *TFPI*:*Xa* complex then binds and inactivates *TF*:*VIIa*, preventing more activation of *Xa*. Alternatively, *TFPI* may bind directly to *Xa* and *VIIa* in the *TF*:*VIIa*:*Xa* complex.

(APC), is also a cofactor of *TFPI* and enhances factor *Xa* inhibition by *TFPI* tenfold.⁵⁷⁻⁵⁹ Because of the inhibitory action of *TFPI*, the *TF*:*VIIa*:*Xa* reaction is short-lived. Once *TFPI* shuts down extrinsic tenase and *Xa*, additional *Xa* and *IXa* production shifts to the intrinsic pathway.⁶⁰ Propagation of coagulation occurs as factor *X* is activated by *IXa*:*VIII* and more factor *IX* is activated by factor *XIa*.

Protein C Regulatory System

During coagulation, thrombin propagates the clot as it cleaves fibrinogen and activates factors *V*, *VIII*, *XI*, and *XIII*. In intact normal vessels, where coagulation would be inappropriate, thrombin avidly binds the EC membrane protein *thrombomodulin* and triggers an essential coagulation regulatory system called the *protein C anticoagulant system*.⁶¹ The protein C system revises thrombin’s function from a procoagulant enzyme to an anticoagulant. EC protein C receptor (*EPCR*) is a transmembrane protein that binds both protein C and *APC* adjacent to the thrombomodulin-thrombin complex and augments the action of thrombin-thrombomodulin at least fivefold in activating protein C to a serine protease (Figure 37-19).^{62,63} *APC* dissociates

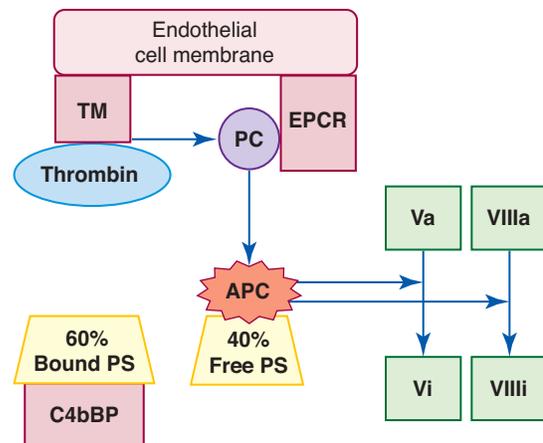


Figure 37-19 Protein C pathway. After binding thrombomodulin (*TM*), thrombin activates protein C (*PC*), bound by endothelial cell protein C receptor (*EPCR*). Free protein S (*PS*) [not bound to C4b binding protein (*C4bBP*)] binds and stabilizes activated protein C (*APC*). The *APC*/protein S complex digests and inactivates factors *Va* (*Vi*, inhibited factor *V*) and *VIIIa* (*VIIIi*, inhibited factor *VIII*).

from EPCR and binds its cofactor, free plasma protein S. The stabilized APC-protein S complex hydrolyzes and inactivates factors Va and VIIIa, slowing or blocking thrombin generation/coagulation.

Protein S, the cofactor that binds and stabilizes APC, is synthesized in the liver and circulates in the plasma in two forms. About 40% of protein S is free, but 60% is covalently bound to the complement control protein C4b-binding protein (C4bBP).⁶⁴ Bound protein S cannot participate in the protein C anticoagulant pathway; only free plasma protein S can serve as the APC cofactor. Protein S-C4bBP binding is of particular interest in inflammatory conditions because C4bBP is an acute phase reactant. When the plasma C4bBP level increases, additional protein S is bound, and free protein S levels become proportionally decreased, which may increase the risk of thrombosis. Chronic acquired or inherited protein C or protein S deficiency or mutations of protein C, protein S, or factor V compromise the normal downregulation of factors Va and VIIIa and may be associated with recurrent venous thromboembolic disease (Chapter 39). Underscoring the importance of the protein C regulatory system, neonates who completely lack protein C have a massive thrombotic condition called *purpura fulminans* and die in infancy unless treated with protein C replacement and anticoagulation.^{65,66}

Antithrombin and Other Serine Protease Inhibitors (Serpins)

Antithrombin (AT) was the first of the coagulation regulatory proteins to be identified and the first to be assayed routinely in the clinical hemostasis laboratory.⁶⁷ Other members of the serpin family include heparin cofactor II, protein Z-dependent protease inhibitor (ZPI), protein C inhibitor, α_1 -protease inhibitor (α_1 -antitrypsin), α_2 -macroglobulin, α_2 -antiplasmin, and PAI-1.⁶⁸

AT is a serine protease inhibitor (serpin) that binds and neutralizes serine proteases, including thrombin (factor IIa) and factors IXa, Xa, XIa, XIIa, prekallikrein, and plasmin.⁶⁹ Heparin cofactor II is a serpin that primarily inactivates thrombin. AT and heparin cofactor II both require heparin for effective anticoagulant activity. In vivo, heparin is available from endothelium-associated mast cell granules or as EC heparan sulfate, a natural glycosaminoglycan that activates AT, although not to the same intensity as therapeutic unfractionated heparin. AT's activity is accelerated 2000-fold by binding to heparin and is the basis for the anticoagulant activity of pharmaceutical heparin. Therapeutically, heparin is administered as unfractionated heparin, low-molecular-weight heparin, or heparin pentasaccharide. Unfractionated heparin consists of chains of greater than 18 sugar units and accelerates inactivation of thrombin through heparin-dependent conformational changes and bridging mechanisms (Figure 37-20). With low-molecular-weight and pentasaccharide heparins lacking long polysaccharide chains for thrombin inactivation, AT preferentially inactivates factor Xa (Chapter 43).

In vivo, antithrombin covalently binds thrombin, forming an inactive thrombin-antithrombin complex (TAT), which is then released from the heparin molecule. Laboratory measurement of TAT is used as an indicator for thrombosis, since it measures both the generation of thrombin and its inhibition.

ZPI, in the presence of its cofactor, protein Z, is a potent inhibitor of factor Xa.^{70,71} ZPI covalently binds protein Z and factor Xa in a complex with Ca^{2+} and phospholipid. Protein Z is a vitamin K-dependent plasma glycoprotein that is synthesized in the liver. Although protein Z has a structure similar to that of the other vitamin K-dependent proteins (factors II, VII, IX, and X and protein C), it lacks an activation site and, like protein S, is nonproteolytic. Protein Z increases the ability of

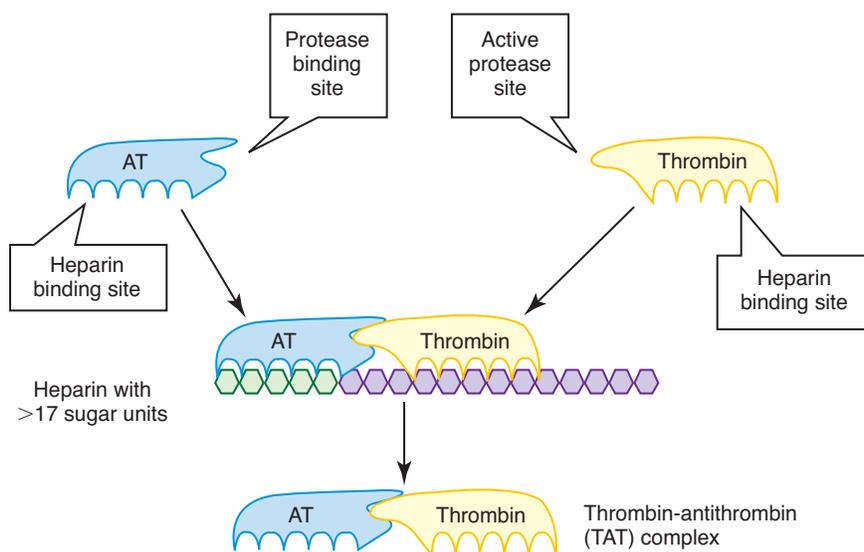


Figure 37-20 Unfractionated heparin potentiates antithrombin-thrombin reaction. Antithrombin (AT) attaches to a specific pentasaccharide sequence in unfractionated heparin. The thrombin binding site for heparin is adjacent to the AT site. The AT is sterically modified to covalently bind and inactivate the thrombin active protease site. Thrombin and AT, covalently bound, release from heparin and form measurable plasma thrombin-antithrombin (TAT) complexes, useful as a marker of coagulation activation.

ZPI to inhibit factor Xa 2000-fold.⁷² ZPI also inhibits factor XIa, in a separate reaction that does not require protein Z, phospholipid, and Ca^{2+} . The inhibition of factor XIa is accelerated twofold by the presence of heparin.

Protein C inhibitor is a nonspecific, heparin-binding serpin that inhibits a variety of proteases, including APC, thrombin, factor Xa, factor XIa, and urokinase. It is found not only in plasma but also in many other body fluids and organs. Depending on its target, it can function as an anticoagulant (inhibits thrombin), as a procoagulant (inhibits thrombin-thrombomodulin and APC), or as a fibrinolytic inhibitor.

The serpins α_1 -protease inhibitor and α_2 -macroglobulin are able to inhibit serine proteases reversibly. See Table 37-11 and the section on fibrinolysis for further information on α_2 -antiplasmin and PAI-1.

FIBRINOLYSIS

Fibrinolysis, the final stage of coagulation (Figure 37-21), begins a few hours after fibrin polymerization and cross-linking. Two activators of fibrinolysis, TPA and UPA, are released in response to inflammation and coagulation. Fibrinolytic proteins assemble on fibrin during clotting. Plasminogen, plasmin, TPA, UPA, and PAI-1 become incorporated into the fibrin clot as they bind to lysine through their “kringle” loops, thereby concentrating and localizing them to the fibrin clot. Fibrinolysis is the systematic, accelerating hydrolysis of fibrin by bound plasmin. TPA and UPA activate fibrin-bound plasminogen several hours after thrombus formation, degrading fibrin and restoring normal blood flow during vascular repair. Again, there is a delicate balance between activators and inhibitors. Excessive fibrinolysis can cause bleeding due to

TABLE 37-11 Proteins of the Fibrinolysis Pathway

Name	Function	Molecular Mass (Daltons)	Half-Life	Mean Plasma Concentration
Plasminogen	Plasma serine protease, plasmin digests fibrin/fibrinogen	92,000	24–26 hr	15–21 mg/dL
Tissue plasminogen activator	Serine protease secreted by activated endothelium, activates plasminogen	68,000	Unknown	4–7 $\mu\text{g/dL}$
Urokinase	Serine protease secreted by kidney, activates plasminogen	54,000	Unknown	—
Plasminogen activator inhibitor-1	Secreted by endothelium, inhibits tissue plasminogen activator	52,000	1 hr	14–28 mg/dL
α_2 -Antiplasmin	Inhibits plasmin	51,000	Unknown	7 mg/dL
Thrombin-activatable fibrinolysis inhibitor	Suppresses fibrinolysis by removing fibrin C-terminal lysine binding sites	55,000	8–10 min	5 $\mu\text{g/mL}$

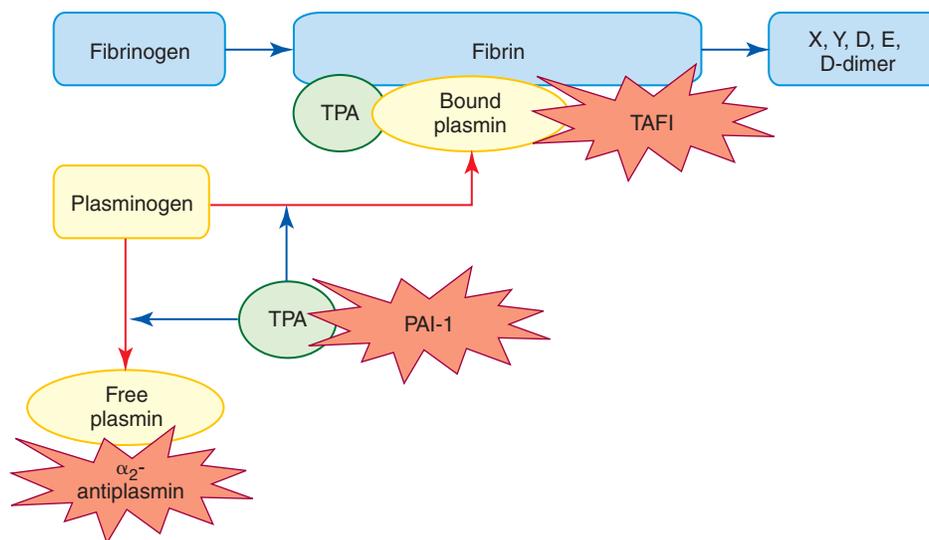


Figure 37-21 Fibrinolysis pathway and inhibitors. Plasminogen and tissue plasminogen activator (TPA) are bound to fibrin during coagulation. TPA converts bound plasminogen to plasmin, which slowly digests fibrin to form fibrin degradation products (FDPs) X, Y, D, E, and D-D (D-dimer). D-dimer is produced from cross-linked fibrin. Free plasmin is neutralized by α_2 -antiplasmin. TPA is neutralized by plasminogen activator inhibitor-1 (PAI-1). Thrombin-activatable fibrinolysis inhibitor (TAFI) inhibits fibrinolysis by cleaving lysine residues on fibrin, preventing the binding of plasminogen, plasmin, and TPA.

premature clot lysis before wound healing is established, whereas inadequate fibrinolysis can lead to clot extension and thrombosis.

Plasminogen and Plasmin

Plasminogen is a 92,000 Dalton plasma zymogen produced by the liver (Table 37-11).^{73,74} It is a single-chain protein possessing five glycosylated loops termed *kringles*. Kringles enable plasminogen, along with activators TPA and UPA, to bind fibrin lysine molecules during polymerization (Figure 37-22). This fibrin-binding step is essential to fibrinolysis. Fibrin-bound plasminogen becomes converted into a two-chain active plasmin molecule when cleaved between arginine at position 561 and valine at position 562 by neighboring fibrin-bound TPA or UPA. Plasmin is a serine protease that systematically digests fibrin polymer by the hydrolysis of arginine-related and lysine-related peptide bonds.⁷⁵ Bound plasmin digests clots and restores blood vessel patency. Its localization to fibrin through lysine binding prevents systemic activity. As fibrin becomes digested, the exposed carboxy-terminal lysine residues bind additional plasminogen and TPA, which further accelerates clot digestion.^{76,77} Free plasmin is capable of digesting plasma fibrinogen, factor V, factor VIII, and fibronectin, causing a potentially fatal primary fibrinolysis. However, plasma α_2 -antiplasmin rapidly binds and inactivates any free plasmin in the circulation.

Plasminogen Activation

Tissue Plasminogen Activator (TPA)

ECs secrete TPA, which hydrolyzes fibrin-bound plasminogen and initiates fibrinolysis. TPA, with two glycosylated kringles, forms covalent lysine bonds with fibrin during polymerization and localizes at the surface of the thrombus with plasminogen, where it begins the digestion process by converting plasminogen to plasmin. Circulating TPA is bound to inhibitors such as PAI-1 and is cleared from plasma. Synthetic recombinant TPAs mimic intrinsic TPA and are a family of drugs used to dissolve pathologic clots that form in venous and arterial thrombotic disease.

Urokinase Plasminogen Activator (UPA)

Urinary tract epithelial cells, monocytes, and macrophages secrete another intrinsic plasminogen activator called *urokinase plasminogen activator*. UPA circulates in plasma at a concentration of 2 to 4 ng/mL and becomes incorporated into the mix of fibrin-bound plasminogen and TPA at the time of thrombus formation. UPA has only one kringles region, does not bind firmly to fibrin, and has a relatively minor physiologic effect. Like TPA, purified UPA preparations are used to dissolve thrombi in myocardial infarction, stroke, and deep vein thrombosis.

Control of Fibrinolysis

Plasminogen Activator Inhibitor 1 (PAI-1)

PAI-1 is the principal inhibitor of plasminogen activation, inactivating both TPA and UPA and thus preventing them from converting plasminogen to the fibrinolytic enzyme plasmin.

PAI-1 is a single-chain glycoprotein serine protease inhibitor and is produced by ECs, megakaryocytes, smooth muscle cells, fibroblasts, monocytes, adipocytes, hepatocytes, and other cell types.^{78,79} Platelets store a pool of PAI-1, accounting for more than half of its availability and for its delivery to the fibrin clot. PAI-1 is present in excess of the TPA concentration in plasma, and circulating TPA normally becomes bound to PAI-1. Only at times of EC activation, such as after trauma, does the level of TPA secretion exceed that of PAI-1 to initiate fibrinolysis. Binding of TPA to fibrin protects TPA from PAI-1 inhibition.⁸⁰ Plasma PAI-1 levels vary widely. PAI-1 deficiency has been associated with chronic mild bleeding due to increased fibrinolysis. PAI-1 is an acute phase reactant and is increased in many conditions, including metabolic syndrome, obesity, atherosclerosis, sepsis, and stroke.⁷⁹ Increased PAI-1 levels correlate with reduced fibrinolytic activity and increased risk of thrombosis.

α_2 -Antiplasmin

α_2 -Antiplasmin (AP) is synthesized in the liver and is the primary inhibitor of free plasmin. AP is a serine protease inhibitor with the unique characteristic of both N- and C-terminal extensions.⁸¹ During thrombus formation, the N-terminus of AP is covalently linked to fibrin by factor XIIIa (Figure 37-22).⁸² The C-terminal contains lysine, which is capable of reacting with the lysine-binding kringles of plasmin. Free plasmin produced by activation of plasminogen can bind either to fibrin, where it is protected from AP because its lysine-binding site is occupied, or to the C-terminus of AP, which rapidly and irreversibly inactivates it. Thus AP with its C-terminal lysine slows fibrinolysis by competing with lysine residues in fibrin for plasminogen binding and by binding directly to plasmin and inactivating it.

The therapeutic lysine analogues, tranexamic acid and ϵ -aminocaproic acid, are similarly antifibrinolytic through their affinity for kringles in plasminogen and TPA. Both inhibit the proteolytic activity of plasmin.

Thrombin-Activatable Fibrinolysis Inhibitor

TAFI is a plasma procarboxypeptidase synthesized in the liver that becomes activated by the thrombin-thrombomodulin complex. This is the same complex that activates the protein C pathway; however, the two functions are independent. Activated TAFI functions as an antifibrinolytic enzyme. It inhibits fibrinolysis by cleaving exposed carboxy-terminal lysine residues from partially degraded fibrin, thereby preventing the binding of TPA and plasminogen to fibrin and blocking the formation of plasmin (Figure 37-22).⁸³ In coagulation factor-deficient states, such as hemophilia, decreased thrombin production may reduce the activation of TAFI, resulting in increased fibrinolysis that contributes to more bleeding. Conversely, in thrombotic disorders, increased thrombin generation may increase the activation of TAFI. The resulting decreased fibrinolysis may contribute further to thrombosis. TAFI also may play a role in regulating inflammation and wound healing.⁸⁴

Fibrin Degradation Products and D-Dimer

Plasmin cleaves fibrin and produces a series of identifiable fibrin fragments: X, Y, D, E, and D-D (Figure 37-23).⁸⁵ Several

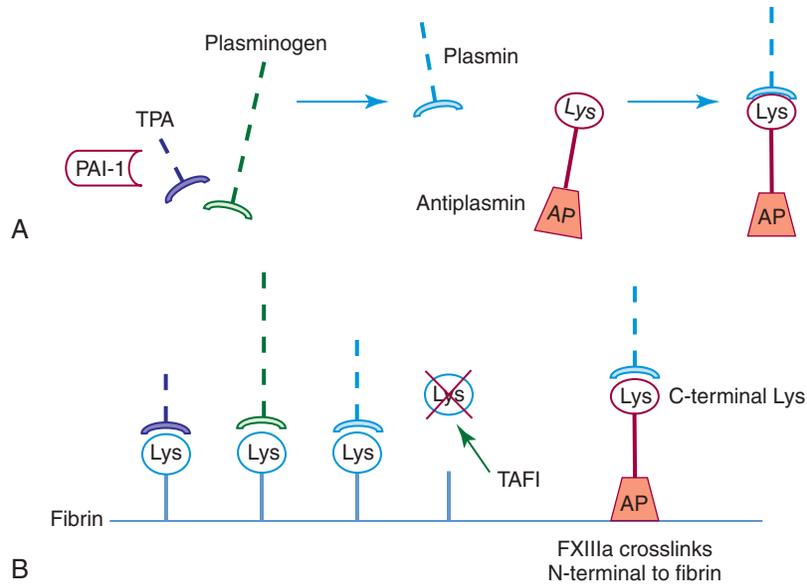


Figure 37-22 Schematic diagram of the action of fibrinolytic proteins. **A**, Tissue plasminogen activator (*TPA*) activates plasminogen to the serine protease, plasmin. *TPA* is inhibited by plasminogen activator inhibitor-1 (*PAI-1*). α_2 -Antiplasmin (*AP*) rapidly inactivates free plasmin. **B**, Fibrinolytic proteins *TPA*, plasminogen, and plasmin bind to fibrin C-terminal lysine (*Lys*) during clotting. Thrombin activatable fibrinolysis inhibitor (*TAFI*) inhibits fibrinolysis by removing the C-terminal *Lys* from fibrin, thereby reducing binding of fibrinolytic proteins. *AP* N-terminus is bound to fibrin by *FXIIIa*. The *AP* C-terminus *Lys* competes with fibrin C-terminus *Lys* to bind plasmin and inactivates it.

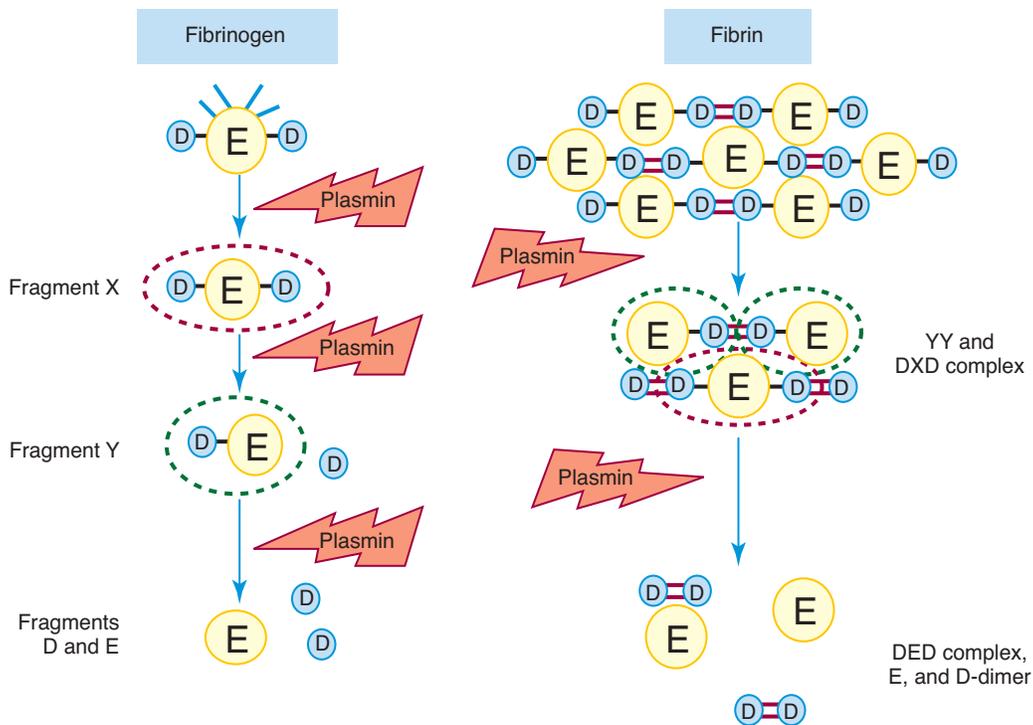


Figure 37-23 Degradation of fibrinogen and fibrin by plasmin. Plasmin systematically degrades fibrinogen and fibrin by digestion of small peptides and cleavage of D-E domains. From fibrinogen, fragment X consists of a central E domain with two D domains (D-E-D); further cleavage produces fragment Y (D-E), with eventual degradation to D and E domains. From cross-linked fibrin, plasmin digestion produces fragment complexes from one or more monomers. D-dimer consists of two D domains from adjacent monomers that have been cross-linked by factor *XIIIa* in the process of fibrin formation (thrombosis).

of these fragments inhibit hemostasis and contribute to hemorrhage by preventing platelet activation and by hindering fibrin polymerization. Fragment X is described as the central E domain with the two D domains (D-E-D), minus some peptides cleaved by plasmin. Fragment Y is the E domain after cleavage of one D domain (D-E). Eventually these fragments are further digested to individual D and E domains.

The D-D fragment, called *D-dimer*, is composed of two D domains from separate fibrin molecules cross-linked by the action of factor XIIIa. Fragments X, Y, D, and E are produced by digestion of either fibrin or fibrinogen by plasmin, but D-dimer is a specific product of digestion of cross-linked fibrin

only and is therefore a marker of thrombosis and fibrinolysis—that is, thrombin, factor XIIIa, and plasmin activation.

The various fragments may be detected by quantitative or semiquantitative immunoassay to reveal fibrinolytic activity. D-dimer is separately detectable by monoclonal antibody for D-dimer antigen, using a wide variety of automated quantitative laboratory immunoassays and other formats including point-of-care tests performed on whole blood.^{86,87} The D-dimer immunoassay is used to identify chronic and acute DIC and to rule out venous thromboembolism in suspected cases of deep venous thrombosis or pulmonary embolism.

SUMMARY

- The vascular intima, platelets, tissue factor–bearing cells, and coagulation and fibrinolytic proteins interact to maintain hemostasis.
- Intact vascular intima prevents coagulation through synthesis of prostacyclin, nitric oxide, TFPI, thrombomodulin, and heparan sulfate.
- Damaged intima promotes coagulation by vasoconstriction, exposure of tissue factor and collagen, and secretion of VWF and other adhesion molecules.
- Platelets function in primary and secondary hemostasis through adhesion, aggregation, and secretion of granular contents.
- Platelets adhere to collagen through VWF and use fibrinogen to aggregate.
- Most coagulation factors are produced in the liver.
- The plasma factors of the prothrombin group (prothrombin; factors VII, IX, and X; protein C; protein S; and protein Z) require vitamin K in their production.
- Plasma coagulation factors include trypsin-like enzymes called serine proteases and cofactors that stabilize the proteases. Factor XIIIa is a transamidase.
- The extrinsic pathway of coagulation consists of the membrane receptor tissue factor and coagulation factors VII, X, V, II, and I. The PT is a screening test for these factors.
- The intrinsic pathway factors are XII, pre-K, HMWK, XI, IX, VIII, X, V, II, and I. The PTT is a screening test for these factors.
- Activation of coagulation pathways produces thrombin, which converts fibrinogen to a fibrin polymer. Thrombin also activates platelets and factors V, VIII, XI, and XIII, and binds to thrombomodulin to activate protein C and TAFI.
- Fibrinogen is cleaved by thrombin to form first fibrin monomer, then fibrin polymer, and finally, when acted on by factor XIIIa, cross-linked fibrin.
- In vivo, coagulation is initiated on tissue factor–bearing cells. TF:VIIa activates factors IX and X, generating enough thrombin to activate platelets and factors V, VIII, and XI. Coagulation proceeds on activated platelet phospholipid membranes with the formation of IXa:VIIIa and Xa:Va complexes, which produces a burst of thrombin that cleaves fibrinogen to fibrin.
- The coagulation pathway is regulated by TFPI, APC, and the serpins, including antithrombin and ZPI. These control proteins prevent thrombosis and confine clotting to the site of injury.
- The fibrinolytic pathway digests the thrombus. Plasminogen is converted to plasmin by TPA. Plasmin degrades fibrin to fragments X, Y, D, and E, and D-dimer. Control proteins are PAI-1, α_2 -antiplasmin, and TAFI.

Now that you have completed this chapter, go back and read again the case study at the beginning and respond to the questions presented.

REVIEW QUESTIONS

Answers can be found in the Appendix.

1. What intimal cell synthesizes and stores VWF?
 - a. Smooth muscle cell
 - b. Endothelial cell
 - c. Fibroblast
 - d. Platelet
2. What subendothelial structural protein triggers coagulation through activation of factor VII?
 - a. Thrombomodulin
 - b. Nitric oxide
 - c. Tissue factor
 - d. Thrombin

3. What coagulation plasma protein should be assayed when platelets fail to aggregate properly?
 - a. Factor VIII
 - b. Fibrinogen
 - c. Thrombin
 - d. Factor X
4. What role does vitamin K play for the prothrombin group factors?
 - a. Provides a surface on which the proteolytic reactions of the factors occur
 - b. Protects them from inappropriate activation by compounds such as thrombin
 - c. Accelerates the binding of the serine proteases and their cofactors
 - d. Carboxylates the factors to allow calcium binding
5. What is the source of fibrinopeptides A and B?
 - a. Plasmin proteolysis of fibrin polymer
 - b. Thrombin proteolysis of fibrinogen
 - c. Proteolysis of prothrombin by factor Xa
 - d. Plasmin proteolysis of cross-linked fibrin
6. What serine protease forms a complex with factor VIIIa, and what is the substrate of this complex?
 - a. Factor VIIa, factor X
 - b. Factor Va, prothrombin
 - c. Factor Xa, prothrombin
 - d. Factor IXa, factor X
7. What protein secreted by endothelial cells activates fibrinolysis?
 - a. Plasminogen
 - b. TPA
 - c. PAI-1
 - d. TAFI
8. What two regulatory proteins form a complex that digests activated factors V and VIII?
 - a. TFPI and Xa
 - b. Antithrombin and protein C
 - c. APC and protein S
 - d. Thrombomodulin and plasmin
9. Coagulation factor VIII circulates bound to:
 - a. VWF
 - b. Factor IX
 - c. Platelets
 - d. Factor V
10. Most coagulation factors are synthesized in:
 - a. The liver
 - b. Monocytes
 - c. Endothelial cells
 - d. Megakaryocytes

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Hemorrhagic Disorders and Laboratory Assessment

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OUTLINE

Bleeding Symptoms

Localized Versus Generalized Hemorrhage

Mucocutaneous Versus Anatomic Hemorrhage

Acquired Versus Congenital Bleeding Disorders

Acquired Coagulopathies

Acute Coagulopathy of Trauma-Shock (ACOTS)

*Liver Disease Coagulopathy
Chronic Renal Failure and Hemorrhage*

Vitamin K Deficiency and Hemorrhage

Autoanti-VIII Inhibitor and Acquired Hemophilia

Acquired von Willebrand Disease

Disseminated Intravascular Coagulation

Congenital Coagulopathies

Von Willebrand Disease

Hemophilia A (Factor VIII Deficiency)

Hemophilia B (Factor IX Deficiency)

Hemophilia C (Rosenthal Syndrome, Factor XI Deficiency)

Other Congenital Single-Factor Deficiencies

OBJECTIVES

After completion of this chapter, the reader will be able to:

1. Distinguish among the causes of localized versus generalized, soft tissue versus mucocutaneous, and acquired versus congenital bleeding.
2. List and interpret laboratory tests that differentiate among acquired hemorrhagic disorders of trauma, liver disease, vitamin K deficiency, and kidney failure.
3. Discuss the laboratory monitoring of therapy for acquired hemorrhagic disorders.
4. Interpret laboratory assay results that diagnose, subtype, and monitor the treatment of von Willebrand disease.
5. Use the results of laboratory tests to identify and monitor the treatment of congenital single coagulation factor deficiencies such as deficiencies of factors VIII, IX, and XI.
6. Explain the principle and rationale for the use of each laboratory test for the detection and monitoring of hemorrhagic disorders.

CASE STUDY

After studying this chapter, the reader will be able to respond to the following case study:

A 55-year-old man comes to the emergency department with epistaxis (uncontrolled nosebleed). He reports that he has “bleeder’s disease” and has had multiple episodes of inflammatory hemarthroses (joint bleeding). Physical examination reveals swollen, immobilized knees; mild jaundice; and an enlarged liver and spleen. CBC results indicate that the patient is anemic and has thrombocytopenia with a platelet count of 74,400/ μL (reference interval, 150,000 to 450,000/ μL). The PT is 18 seconds (reference interval, 12 to 14 seconds), and the PTT is 43 seconds (reference interval, 25 to 35 seconds).

1. What is the most likely diagnosis?
2. What treatment does the patient need?

BLEEDING SYMPTOMS

Hemorrhage is severe bleeding that requires physical intervention. Hemorrhage may be *localized* or *general*, *acquired* or *congenital*. To establish the cause of a patient’s tendency to bleed or a bleeding event, the physician obtains a complete patient

and family history and performs a physical examination before ordering diagnostic laboratory tests.¹

Localized Versus Generalized Hemorrhage

Bleeding from a single location commonly indicates injury, infection, tumor, or an isolated blood vessel defect and is

*The author acknowledges the substantial contributions of Marisa B. Marques, MD, to Chapter 41 of the third edition of this textbook, many of which remain in this edition.

called *localized bleeding* or *localized hemorrhage*. An example of localized bleeding is an inadequately cauterized or ineffectively sutured surgical site. Localized bleeding seldom implies a blood vessel defect, a qualitative platelet defect, a reduced platelet count (thrombocytopenia), or a coagulation factor deficiency.

Bleeding from multiple sites, spontaneous and recurring bleeds, or a hemorrhage that requires physical intervention and transfusions is called *generalized bleeding*. Generalized bleeding is potential evidence for a disorder of *primary hemostasis* such as blood vessel defects, platelet defects, or thrombocytopenia, or *secondary hemostasis* characterized by single or multiple coagulation factor deficiencies.

Mucocutaneous Versus Anatomic Hemorrhage

Generalized bleeding may exhibit a *mucocutaneous* (*systemic*) or a soft tissue (*anatomic*) pattern. Mucocutaneous hemorrhage may appear as *petechiae*, pinpoint hemorrhages into the skin (Figure 40-1, A); *purpura*, purple lesions of the skin greater than 3 mm caused by extravasated (seeping) red blood cells (RBCs) (Figure 40-1, B); or *ecchymoses* (bruises) greater than 1 cm typically seen after trauma (Fig. 40-1, C).² The unprovoked presence of more than one such lesion may indicate a disorder of primary hemostasis. Other symptoms of a primary hemostasis defect include bleeding from the gums, epistaxis (uncontrolled nosebleed), hematemeses (vomiting of blood), and menorrhagia (profuse menstrual flow). Although nosebleeds are common and mostly innocent, especially in children, they suggest a primary hemostatic defect when they occur repeatedly, last longer than 10 minutes, involve both nostrils, or require physical intervention or transfusions.

Mucocutaneous hemorrhage tends to be associated with thrombocytopenia (platelet count lower than 150,000/ μ L; Chapter 40), qualitative platelet disorders (Chapter 41), von Willebrand disease (VWD), or vascular disorders such as scurvy or telangiectasia (Chapters 13 and 37). A careful history and physical examination distinguish between mucocutaneous and anatomic bleeding; this distinction helps direct investigative laboratory testing and subsequent treatment.

Anatomic (soft tissue) hemorrhage is seen in acquired or congenital defects in secondary hemostasis, or *plasma coagulation factor* deficiencies (coagulopathies).³ Examples of anatomic bleeding include recurrent or excessive bleeding after minor trauma, dental extraction, or a surgical procedure. In such cases, hemorrhage may immediately follow a primary event, but it is often delayed or recurs minutes or hours after the event. Anatomic bleeding episodes may even be spontaneous. Most anatomic bleeds are internal, such as bleeds into joints, body cavities, muscles, or the central nervous system, and may initially have few visible signs. Because joint bleeds (*hemarthroses*) cause swelling and acute pain, they may not be immediately recognized as hemorrhages, although experienced hemophilia patients usually recognize the symptoms at their outset. Repeated hemarthroses cause painful swelling and

inflammation, and they may culminate in permanent cartilage damage that immobilizes the joint. Bleeds into soft tissues such as muscle or fat may cause nerve compression and subsequent temporary or permanent loss of function.⁴ When bleeding involves body cavities, it causes symptoms related to the organ that is affected. Bleeding into the central nervous system, for instance, may cause headaches, confusion, seizures, and coma and is managed as a medical emergency. Bleeds into the kidney may present as *hematuria* and may be associated with acute kidney failure.

Hemostasis laboratory testing is essential whenever a mucocutaneous or soft tissue disorder is suspected. **Box 38-1** lists symptoms that suggest generalized hemorrhagic disorders. Besides a complete blood count that includes a platelet count, most laboratory directors offer the prothrombin time (PT), partial thromboplastin time (PTT), and fibrinogen assay (**Table 38-1**).⁵ In 2000, laboratory practitioners began to add *thromboelastography* performed using the Thromboelastograph® (TEG, Haemoscope Corporation, Niles, IL) or subsequently *thromboelastometry* (TEM) using the rotational thromboelastometer (ROTEM®, Pentapharm GmbH, Munich, Germany; FDA-cleared 2011) (Chapter 44). TEG and TEM are coagulometers that measure

BOX 38-1 Generalized Bleeding Signs Heralding a Possible Hemostatic Defect

- Purpura—recurrent, chronic bruising in multiple locations; called *petechiae* when less than 3 mm in diameter, *ecchymoses* when greater than 1 cm in diameter
- Epistaxis—nosebleeds that are recurrent, last longer than 10 minutes, or require physical intervention
- Recurrent or excessive bleeding from trauma, surgery, or dental extraction
- Bleeding into multiple body cavities, joints, or soft tissue
- Simultaneous hemorrhage from several sites
- Menorrhagia (menstrual hemorrhage)
- Bleeding that is delayed or recurrent
- Bleeding that is inappropriately brisk
- Bleeding for no apparent reason
- Hematemesis (vomiting of blood)

TABLE 38-1 Screening Tests for a Generalized Hemostatic Disorder

Test	Assesses
Hemoglobin, hematocrit; reticulocyte count	Anemia associated with chronic bleeding; bone marrow response
Platelet count	Thrombocytopenia
Prothrombin time (PT)	Deficiencies of factors II (prothrombin), V, VII, or X (clotting time prolonged)
Partial thromboplastin time (PTT)	Deficiencies of all factors except VII and XIII (clotting time prolonged)
Thrombin time or fibrinogen assay	Hypofibrinogenemia and dysfibrinogenemia

whole blood clotting.⁶ Both report clot onset, clot strength, and fibrinolysis within 15 minutes of blood collection. Experienced laboratory practitioners must interpret TEG or TEM results.

Acquired Versus Congenital Bleeding Disorders

Liver disease, kidney failure, chronic infections, autoimmune disorders, obstetric complications, dietary deficiencies such as vitamin C or vitamin K deficiency, blunt or penetrating trauma, and inflammatory disorders may all associate with bleeding. If a patient's bleeding episodes begin after childhood, are associated with some disease or physical trauma, and are not duplicated in relatives, they are probably acquired, not congenital. When an adult patient comes for treatment of generalized hemorrhage, the physician first looks for an underlying disease or event and records a personal and family history (Box 38-2). The important elements of patient history are age, sex, current or past pregnancy, any systemic disorders such as diabetes or cancer, trauma, and exposure to drugs, including prescription drugs, over-the-counter drugs, alcohol abuse, or drug abuse. The physician determines the trigger, location, and volume of bleeding, and then orders laboratory assays (Table 38-1). These tests take on clinical significance when the history and physical examination already have established the existence of abnormal bleeding. Owing to their propensity for false positives, they are not effective when employed indiscriminately as screens for healthy individuals (Chapter 5).⁷

Congenital hemorrhagic disorders are uncommon, occurring in fewer than 1 per 100 people, and are usually diagnosed in infancy or during the first years of life.⁸ There may be relatives with similar symptoms. Congenital bleeding disorders lead to repeat hemorrhages that may be spontaneous or may occur following minor injury or in unexpected locations, such as joints, body cavities, retinal veins and arteries, or the central nervous system. Patients with mild congenital hemorrhagic disorders may have no symptoms until they reach adulthood or experience some physical challenge, such as trauma, dental extraction, or a surgical procedure. The most common congenital deficiencies are VWD, factor VIII and IX deficiencies (hemophilia A and B), and platelet function disorders. Inherited deficiencies of fibrinogen, prothrombin, and factors V, VII, X, XI, and XIII are rare.

BOX 38-2 Indications of Congenital Hemorrhagic Disorders

- Relatives with similar bleeding symptoms
- Onset of bleeding in infancy or childhood
- Bleeding from umbilical cord or circumcision wound
- Repeated hemorrhages in childhood, adulthood
- Hemorrhage into joints, central nervous system, soft tissues, peritoneum

ACQUIRED COAGULOPATHIES

Far more patients experience acquired bleeding disorders secondary to trauma, drug exposure, or chronic disease than possess inherited coagulopathies. Chronic disorders commonly associated with bleeding are *liver disease*, *vitamin K deficiency*, and *renal failure*. In all cases, laboratory test results are necessary to confirm the diagnosis and guide the management of acquired hemorrhagic events.^{1,9}

Acute Coagulopathy of Trauma-Shock

In North America, unintentional injury is the leading cause of death among those aged 1 to 45 years. The total rises when statisticians include self-inflicted, felonious, and combat injuries. In the United States alone, trauma causes 93,000 deaths per year.¹⁰ Severe neurologic displacement accounts for 50% of trauma deaths, with most occurring before the patient arrives at the hospital; however, of initial survivors, 20,000 die of hemorrhage within 48 hours.¹¹ *Acute coagulopathy of trauma-shock* (ACOTS) accounts for most fatal hemorrhage, and 3000 to 4000 of these deaths are preventable. Coagulopathy is defined as any hemostasis deficiency, and ACOTS is triggered by the combination of injury-related *acute inflammation*, *platelet activation*, *tissue factor release*, *hypothermia*, *acidosis*, and *hypoperfusion* (poor distribution of blood to tissues caused by low blood pressure), all of which are elements of systemic shock. For in-transit fluid resuscitation, colloid plasma expanders such as 5% dextrose are used by most emergency medical technicians to counter hypoperfusion, though in 2013 some trauma specialists advocate for plasma instead of colloids as a means to control coagulopathy.¹² Colloids dilute plasma procoagulants, which become further diluted by emergency department transfusion of RBCs. Although colloids and RBCs are essential, they intensify the coagulopathy, as does subsequent surgical intervention.

Massive transfusion, defined as administration of more than 3 RBC units within 1 hour or 8 to 10 units within 24 hours, is essential in otherwise healthy trauma victims when the systolic blood pressure is below 110 mm Hg, pulse is greater than 105 beats/min, pH is less than 7.25, hematocrit is below 32%, hemoglobin is below 10 g/dL, and PT is prolonged to more than 1.5 times the mean of the reference interval or generates an international normalized ratio (INR) of 1.5.¹³

Acute Coagulopathy of Trauma-Shock Management: RBCs

According to the American Society of Anesthesiologists surgical practice guidelines, RBC transfusions are required when the hemoglobin is less than 6.0 g/dL and are contraindicated when the hemoglobin is greater than 10.0 g/dL.¹⁴ For hemoglobin concentrations between 6.0 and 10.0 g/dL, the decision to transfuse is based upon the acuity of the patient's condition as determined by *physical evidence of blood loss*; *current blood loss rate*; *blood pressure*; *arterial blood gas values, especially pH and oxygen saturation*; *urine output*; and *laboratory evidence for*

coagulopathy provided there is time for specimens to be collected and laboratory assays completed.

Acute Coagulopathy of Trauma-Shock

Management: Plasma

Plasma remains a key component of ACOTS management. Before 2005, donor services centrifuged donor blood units and separated and froze the supernatant plasma within 8 hours of collection, yielding fresh-frozen plasma (FFP). Most donor services now separate plasma within 24 hours of donor collection, officially naming the product FP-24 instead of FFP; however, laboratory practitioners, nurses, and physicians still say and write “FFP” from habit.¹⁵ FP-24 or FFP may also be thawed and stored at 1° C to 6° C for up to 5 days, a product called “thawed plasma.”¹⁶ Von Willebrand factor (VWF) and coagulation factor V and VIII activities decline to approximately 60% after 5 days of refrigerator storage, so thawed plasma may be ineffective in von Willebrand disease or hemophilia. High-volume trauma centers and mobile emergency services may maintain a small inventory of thawed plasma ready for emergency administration.

Frozen plasma is thawed, warmed to 37° C, and transfused when there is microvascular bleeding and the PT is prolonged to greater than 1.5 times the mean of the PT reference interval or if the PTT is prolonged to greater than 2 times the mean of the PTT reference interval. Plasma is also transfused when the patient is known to have a preexisting single coagulation factor deficiency such as factor VIII deficiency and no factor concentrate is available, when there has been significant volume replacement with colloid plasma expanders and RBCs, and when hemorrhage is complicated by or caused by *Coumadin overdose* (Chapter 43).

Most transfusion service directors recommend that 1 plasma unit be administered per 4 RBC units to retain coagulation stability; however, evidence from retrospective studies on battlefield casualties in Iraq and Afghanistan have led to consideration of a ratio of 1 plasma unit to each RBC unit. The 1:1 ratio appears to provide better coagulation stability, and many transfusion services have modified their massive transfusion protocols to approximate the new ratios.^{17,18}

The standard adult plasma dosage is 10 to 15 mL/kg in continuous infusion. Theoretically, plasma should be transfused until 30% coagulation factor activity has been reached for all factors; however, the actual volume that may be administered is limited by the risk of *transfusion-associated circulatory overload* (TACO).¹⁹

TACO and *transfusion-related acute lung injury* (TRALI) are potential adverse effects of plasma administration.²⁰ TACO and TRALI can lead to potentially fatal acute *adult respiratory distress syndrome* (ARDS). Plasma therapy may also cause *thrombosis*, *anaphylaxis*, and *multiple organ failure*.

Acute Coagulopathy of Trauma-Shock Management: Platelet Concentrate

During surgery, coagulopathy is assessed based on *microvascular bleeding*, which is evaluated by estimating the volume of blood that fills suction canisters, surgical sponges, and surgical

drains. *Platelet concentrate* is ordered when the platelet count is less than 50,000/ μ L or higher when the surgeon anticipates blood loss.²¹ Platelet concentrate therapy is generally ineffective when the patient is known to have *immune thrombocytopenic purpura*, *thrombotic thrombocytopenic purpura*, or *heparin-induced thrombocytopenia*. In patients with these conditions, therapeutic platelets are rapidly consumed, and their administration may therefore be contraindicated, although they may provide temporary rescue. Platelet concentrate is never ordered when the platelet count is greater than 100,000/ μ L. Platelet administration may be necessary when the platelet count is between 50,000 and 100,000/ μ L and there is bleeding into a confined space such as the brain or eye; if the patient is taking antiplatelet agents, aspirin, clopidogrel, prasugrel, or ticagrelor; if there is a known platelet disorder such as a release defect, Glanzmann thrombasthenia, or Bernard-Soulier syndrome (Chapters 40 and 41); or if the surgery involves cardiopulmonary bypass, which suppresses platelet activity.¹⁴

Acute Coagulopathy of Trauma-Shock Management: Components and Concentrates

In an effort to reduce the risk of TACO and TRALI, improve patient outcomes, and conserve resources, transfusion service directors employ components and concentrates to augment or even replace plasma administration. Activated prothrombin complex concentrate (PCC, FEIBA® [factor eight inhibitor bypassing activity], Baxter Healthcare Corp., Deerfield, IL, or Autoplex T®, Nabi Biopharmaceuticals, Inc., Boca Raton, FL) may be used at a dosage of 50 units/kg every 12 hours, not to exceed 200 units/kg in 24 hours.^{22,23} The dose-response relationship of FEIBA or Autoplex T varies among recipients, and because both contain activated coagulation factors, their use raises the risk of disseminated intravascular coagulation (DIC). Nonactivated PCCs such as four-factor concentrate Kcentra® (CLS Behring, King of Prussia, PA) are safer and may also be employed. Kcentra was FDA-cleared in 2013 to treat hemorrhage in Coumadin overdose, but its use in ACOTS is off-label (not FDA cleared).^{24,25}

PCCs, either activated or nonactivated, may be used in conjunction with the antifibrinolytic lysine analogue *tranexamic acid* (TXA, Cyklokapron®, Pharmacia & Upjohn Co., Division of Pfizer Inc., New York, NY).²⁶ TXA may be infused at a loading dose of 1 g administered over 10 minutes followed by infusion of 1 g over 8 hours.²⁷ First FDA-cleared in 1986 to prevent bleeding in hemophilic patients who are about to undergo invasive procedures, TXA is effective for ACOTS, but this is an off-label use.

Administration of *cryoprecipitate* or *fibrinogen concentrate* (RiaSTAP®, CLS Behring, King of Prussia, PA) is indicated when there is microvascular bleeding and the fibrinogen concentration is less than 100 mg/dL.²⁸ A 15 to 20 mL unit of cryoprecipitate provides 150 to 250 mg of fibrinogen, and the risk of TACO is lower than that with colloids or plasma. The fibrinogen concentrate dose is 70 mg/kg of body weight infused at less than 5 mL/minute, and a target fibrinogen level of 100 mg/dL should be maintained. Von Willebrand factor and factor VIII concentrates may also be used when

the patient is deficient. Plasma and PCCs may all provide reduced factor VIII levels.^{29,30}

Recombinant activated coagulation factor VII (rVIIa, NovoSeven, Novo Nordisk Inc., Princeton, NJ) was FDA-cleared in 1999 for treating hemophilia A or B when factor VIII or factor IX inhibitors are present, respectively; its application in the treatment of ACOTS is off-label. A NovoSeven dosage of 30 µg/kg is rapidly effective in halting microvascular hemorrhage in nonhemophilic trauma victims, and NovoSeven does not cause DIC.^{9,31-33} However, one study found a possible link between off-label NovoSeven use and arterial and venous thrombosis in patients with existing thrombotic risk factors.³⁴

Acute Coagulopathy of Trauma-Shock: Monitoring Therapy

A skilled operator employing TEG or TEM technology may monitor plasma, PCCs, activated PCC, four-factor PCC, TXA, and rVIIa. Fibrinogen concentrate or cryoprecipitate efficacy may be measured using the fibrinogen assay. Also, laboratory directors characteristically advise surgeons and emergency department physicians to monitor the effectiveness of all ACOTS therapy indirectly by checking for the correction of platelet count, PT, and PTT to within their respective reference intervals. Platelet aggregometry may be used to measure post-therapy platelet function, and coagulation factor assays are valuable as follow-ups to PT and PTT to determine if the target activity of 30% has been met. While PT, PTT, platelet count, and platelet function assays are accepted approaches, TEG and TEM provide immediate feedback and may be more sensitive to small physiologic improvements.³⁵ Once ACOTS has been stabilized, additional hemostasis-related therapy is seldom required.

Liver Disease Coagulopathy

The bleeding associated with liver disease may be localized or generalized, mucocutaneous or anatomic. Enlarged and collateral esophageal vessels called *esophageal varices* are a complication of chronic alcoholic cirrhosis; hemorrhaging from varices is localized bleeding, not a coagulopathy, though often fatal. Mucocutaneous bleeding occurs in liver disease-associated thrombocytopenia, often accompanied by decreased platelet function. Soft tissue bleeding is the consequence of procoagulant dysfunction and deficiency.

Procoagulant Deficiency in Liver Disease

The liver produces nearly all of the plasma coagulation factors and regulatory proteins. Hepatitis, cirrhosis, obstructive jaundice, cancer, poisoning, and congenital disorders of bilirubin metabolism may suppress the synthetic function of hepatocytes, reducing either the concentrations or activities of the plasma coagulation factors to below hemostatic levels (less than 30% of normal).

Liver disease predominantly alters the production of the vitamin K-dependent factors II (prothrombin), VII, IX, and X and control proteins C, S, and Z. In liver disease, these seven factors are produced in their *des-γ-carboxyl* forms, which cannot participate in coagulation (Chapter 37). At the onset of liver

disease, factor VII, which has the shortest plasma half-life at 6 hours, is the first coagulation factor to exhibit decreased activity. Because the PT is particularly sensitive to factor VII activity, it is characteristically prolonged in mild liver disease, serving as a sensitive early marker.³⁶ Vitamin K deficiency caused by dietary insufficiency independent of liver disease produces a similar effect on the PT (see the case study in this chapter).

Declining coagulation factor V activity is a more specific marker of liver disease than deficient factor II, VII, IX, or X because factor V is non-vitamin K dependent and is not affected by dietary vitamin K deficiency. The factor V activity assay, performed in conjunction with the factor VII assay, may be used to distinguish liver disease from vitamin K deficiency.³⁷

Fibrinogen is an acute phase reactant that is frequently elevated in early or mild liver disease. Moderately and severely diseased liver produces fibrinogen that is coated with excessive sialic acid, a condition called *dysfibrinogenemia*, in which the fibrinogen functions poorly. Dysfibrinogenemia causes generalized soft tissue bleeding associated with a prolonged thrombin time and an exceptionally prolonged reptilase clotting time.³⁸ In end-stage liver disease, the fibrinogen level may fall to less than 100 mg/dL, which is a mark of liver failure.³⁹

VWF and factors VIII and XIII are acute phase reactants that may be unaffected or elevated in mild to moderate liver disease.^{40,41} In contrast to the other coagulation factors, VWF is produced from endothelial cells and megakaryocytes and is stored in endothelial cells and platelets.

Platelet Abnormalities in Liver Disease

Moderate *thrombocytopenia* occurs in a third of patients with liver disease. Platelet counts of less than 150,000/µL may result from sequestration and shortened platelet survival associated with portal hypertension and resultant hepatosplenomegaly.⁴² In alcoholism-related hepatic cirrhosis, acute alcohol toxicity also suppresses platelet production. Platelet aggregation and secretion properties are often suppressed; this is reflected in reduced platelet aggregometry and lumiaggregometry results (Chapter 42). Occasionally platelets are hyperreactive. Aggregometry may be used to predict bleeding and thrombosis risk.⁴³

Disseminated Intravascular Coagulation in Liver Disease

Chronic or compensated DIC (Chapter 39) is a significant complication of liver disease that is caused by decreased liver production of regulatory antithrombin, protein C, or protein S and by the release of activated procoagulants from degenerating liver cells. The failing liver cannot clear activated coagulation factors. In primary or metastatic liver cancer, hepatocytes may also produce procoagulant substances that trigger chronic DIC, leading to ischemic complications.

In acute, uncompensated DIC, the PT, PTT, and thrombin time are prolonged; the fibrinogen level is reduced to less than 100 mg/dL; and fibrin degradation products, including D-dimers, are significantly increased.⁴⁴ If the DIC is chronic and compensated, the only elevated test result may be the

D-dimer assay value, a hallmark of unregulated coagulation and fibrinolysis. Although DIC can be resolved only by removing its underlying cause, its hemostatic deficiencies may be corrected temporarily by administering RBCs, plasma, activated or nonactivated PCC, TXA, platelet concentrates, or antithrombin concentrates.⁴⁵

Hemostasis Laboratory Tests in Liver Disease

The PT, PTT, thrombin time, fibrinogen concentration, platelet count, and D-dimer concentration are used to characterize the hemostatic abnormalities in liver disease (Table 38-2). Factor V and VII assays may be used in combination to differentiate liver disease from vitamin K deficiency. Both factors are decreased in liver disease, but factor V is not decreased in vitamin K deficiency.

Plasminogen deficiency and an elevated D-dimer confirm systemic fibrinolysis. The *reptilase time* test occasionally may be useful to confirm dysfibrinogenemia. This test duplicates the thrombin time test except that venom of the reptile *Bothrops atrox* (common lancehead viper) is substituted for thrombin reagent. The *Bothrops* venom triggers fibrin

polymerization by cleaving fibrinopeptide A but not fibrinopeptide B from the fibrinogen molecule. The subsequent polymerization is slowed by structural defects, which prolong the time interval to clot formation. The reptilase time test is unaffected by standard unfractionated heparin therapy and can be used to assess fibrinogen function even when there is heparin in the sample.⁴⁶

Hemostatic Treatment to Resolve Liver Disease–Related Hemorrhage

Oral or intravenous vitamin K therapy may correct the bleeding associated with nonfunctional des- γ -carboxyl factors II (prothrombin), VII, IX, and X; however, the therapeutic effect of vitamin K is short-lived compared to its effect in dietary vitamin K deficiency because of the liver's impaired synthetic ability. In severe liver disease, plasma transfusion provides all of the coagulation factors in hemostatic concentrations, although VWF and factors V and VIII may be reduced. Owing to its small concentration and short half-life of factor VII, plasma is unlikely to return the PT to within the reference interval.

A unit of plasma has a volume of 200 to 280 mL. The typical adult plasma dose for liver disease is 2 units, but the dose varies widely, depending on the indication and the ability of the patient's cardiac and renal system to rapidly excrete excess fluid. TACO is likely to occur when 30 mL/kg has been administered, but it may occur with even smaller volumes in patients with compromised cardiac or kidney function.

If the fibrinogen level is less than 50 mg/dL, spontaneous bleeding is imminent, and cryoprecipitate or fibrinogen concentrate may be selected for therapy. Plasma and cryoprecipitate present a theoretical risk of virus transmission, as do other untreated single-donor biologic blood products, and allergic transfusion reactions are more common with plasma-containing products. Other therapeutic options for patients with liver disease–related bleeding are platelet concentrates, PCC, antithrombin concentrate (Thrombate III, Telacris® Biotherapeutics, Inc., Research Triangle Park, NC), rFVIIa, and TXA.

TABLE 38-2 Hemostasis Laboratory Tests in Liver Disease

Assay	Interpretation
Fibrinogen	>400 mg/dL in early, mild liver disease; <200 mg/dL in moderate to severe liver disease, causing hypofibrinogenemia or dysfibrinogenemia
Thrombin time	Prolonged in dysfibrinogenemia, hypofibrinogenemia, elevation of fibrin degradation products, and therapy with unfractionated heparin
Reptilase time	Prolonged in hypofibrinogenemia, significantly prolonged in dysfibrinogenemia; not affected by heparin; assay rarely used
Prothrombin time (PT)	Prolonged even in mild liver disease due to des- γ -carboxyl factors replacing normal factors II (prothrombin), VII, IX, and X. Report PT in seconds, not international normalized ratio (INR), when testing for liver disease.
Partial thromboplastin time (PTT)	Mildly prolonged in severe liver disease due to disseminated intravascular coagulation (DIC) or des- γ -carboxyl factors II (prothrombin), IX, and X
Factor V assay	Factor V level becomes reduced in liver disease, but is unaffected by vitamin K deficiency so the factor V level helps distinguish the conditions
Platelet count	Mild thrombocytopenia, platelet count <150,000/ μ L
Platelet aggregometry	Mild suppression of platelet aggregation and secretion in response to most agonists
D-dimer	>240 ng/mL by quantitative assay

Chronic Renal Failure and Hemorrhage

Chronic renal failure of any cause is often associated with *platelet dysfunction* and *mild to moderate mucocutaneous bleeding*.⁴⁷ Platelet adhesion to blood vessels and platelet aggregation are suppressed, perhaps because the platelets become coated by *guanidinosuccinic acid* or dialyzable *phenolic compounds*.⁴⁸ Decreased RBC mass (anemia) and thrombocytopenia contribute to the bleeding and may be corrected with dialysis, *erythropoietin* or RBC transfusions, and interleukin-11 therapy.⁴⁹⁻⁵²

Hemostasis activation syndromes that deposit fibrin in the renal microvasculature reduce glomerular function. Examples of such disorders are DIC, *hemolytic uremic syndrome*, and thrombotic thrombocytopenic purpura. Although these are by definition thrombotic disorders, they invariably cause thrombocytopenia, which may lead to mucocutaneous bleeding. Fibrin also may be deposited in renal transplant rejection and in the glomerulonephritis syndrome of systemic lupus erythematosus. This may be associated with a rise in quantitative plasma D-dimer, thrombin-antithrombin complexes, or prothrombin

fragments 1 + 2, which are markers of coagulation activation (Chapter 42).⁵³

Laboratory tests for bleeding in renal disease provide only modest information with little predictive or management value. The bleeding time test may be prolonged, but it is too unreliable to provide an accurate diagnosis or to assist in monitoring treatment.⁵⁴ Platelet aggregometry test results may predict bleeding.⁵⁵ The PT and PTT are expected to be normal.

Management of renal failure-related bleeding typically focuses on the severity of the hemorrhage without reliance on laboratory test results. Renal dialysis temporarily activates platelets and may ultimately improve platelet function, particularly when anemia is well controlled.^{56,57} Desmopressin acetate may be administered intravenously (DDAVP) or intranasally (Stimate®, CSL Behring, King of Prussia, PA) to increase the plasma concentration of high-molecular-weight multimers of VWF, which also aids platelet adhesion and aggregation.⁵⁸ Patients with renal failure should not take aspirin, clopidogrel, prasugrel, ticagrelor, or other platelet inhibitors, because these drugs increase the risk of hemorrhage.

Nephrotic Syndrome and Hemorrhage

Nephrotic syndrome is a state of increased glomerular permeability associated with a variety of conditions, such as chronic glomerulonephritis, diabetic glomerulosclerosis, systemic lupus erythematosus, amyloidosis, and renal vein thrombosis.⁵⁹ In nephrotic syndrome, low-molecular-weight proteins are lost through the glomerulus into the glomerular filtrate and the urine. Coagulation factors II (prothrombin), VII, IX, X, and XII have been detected in the urine, as have the coagulation regulatory proteins antithrombin and protein C. In 25% of cases, loss of regulatory proteins takes precedence over loss of procoagulants and leads to a tendency toward venous thrombosis.

Vitamin K Deficiency and Hemorrhage

Vitamin K is ubiquitous in foods, especially green leafy vegetables, and the daily requirement is small, so pure dietary deficiency is rare. Body stores are limited, however, and become exhausted when the usual diet is interrupted, as when patients are fed only with parenteral (intravenous) nutrition for an extended period or when people embark upon fad diets. Also, because vitamin K is fat soluble and requires bile salts for absorption, biliary duct obstruction (*atresia*), fat malabsorption, and chronic diarrhea may cause vitamin K deficiency. Broad-spectrum antibiotics that disrupt normal gut flora may cause a slight reduction because they destroy bacteria that produce vitamin K. The degree of reduction is insignificant when the diet is otherwise normal but may become an important issue when the patient is receiving only parenteral nutrition.⁶⁰

Hemorrhagic Disease of the Newborn Caused by Vitamin K Deficiency

Because of their sterile intestines and the minimal concentration of vitamin K in human milk, newborns are constitutionally vitamin K deficient.⁶¹ Hemorrhagic disease of the newborn was common in the United States before routine administration of vitamin K to infants was legislated in the 1960s, and it

still occurs in developing countries. The activity levels of factors II (prothrombin), VII, IX, and X are lower in normal newborns than in adults, and premature infants have even lower concentrations of these factors. Breastfeeding prolongs the deficiency because passively acquired maternal antibodies delay the establishment of gut flora.

Vitamin K Antagonists: Coumadin

The γ -carboxylation cycle of coagulation factors is interrupted by coumarin-type oral anticoagulants such as Coumadin (warfarin) that disrupt the vitamin K epoxide reductase and vitamin K quinone reductase reactions (Figure 38-1).⁶² In this situation, the liver releases dysfunctional des- γ -carboxyl factors II (prothrombin), VII, IX, and X, and proteins C, S, and Z; these inactive forms are called *proteins in vitamin K antagonism* (PIVKA). Therapeutic overdose or the accidental or felonious administration of warfarin-containing rat poisons may result in moderate to severe hemorrhage because of the lack of functional factors. The effect of *brodifacoum* or “superwarfarin,” often used as a rodenticide, lasts for weeks to months, and treatment of poisoning with this substance requires repeated administration of vitamin K with follow-up PT monitoring.⁶³ Coumadin overdose is the single most common reason for hemorrhage-associated emergency department visits.

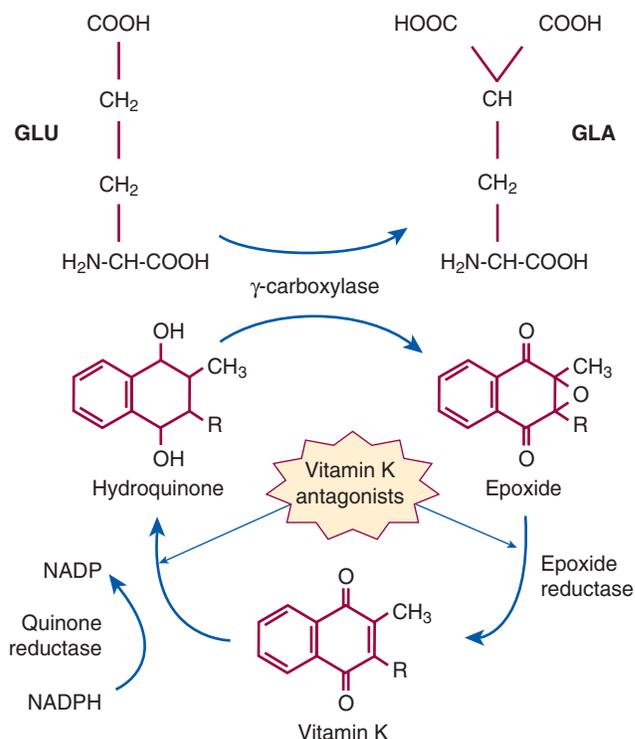


Figure 38-1 Glutamic acid (GLU) gains a carboxyl group to become γ -carboxyglutamic acid (GLA) when catalyzed by γ -carboxylase. This post-translational modification enables the vitamin K-dependent coagulation factors II (prothrombin), VII, IX, and X and proteins C, S, and Z to bind ionic calcium necessary for normal coagulation. Vitamin K donates the carboxyl group through the quinone reductase pathway. Warfarin inactivates quinone reductase and epoxide reductase to prevent carboxylation. *NADP*, Nicotinamide adenine dinucleotide phosphate; *NADPH*, reduced form of nicotinamide adenine dinucleotide phosphate; *R*, any side chain.

Detection of Vitamin K Deficiency or Proteins in Vitamin K Antagonism

Clinical suspicion of vitamin K deficiency is supported by a prolonged PT with or without a prolonged PTT. In PT and PTT mixing studies, if pooled platelet-free normal plasma (NP; Cryocheck, Precision BioLogic, Inc., Dartmouth, Nova Scotia) is combined with patient plasma, the mixture yields normal (corrected) PT and PTT results, which indicates that factor deficiencies were the cause of the prolonged screening test times. Specific single-factor assays always detect low factor VII because of its short half-life, followed in turn by decreases in factors IX, X, and II (prothrombin).

The standard therapy for vitamin K deficiency is oral—or, in an emergency, intravenous—vitamin K. Because synthesis of functional vitamin K-dependent coagulation factors requires at least 3 hours, in the case of severe bleeding, plasma, activated or nonactivated PCC, four-factor PCC, or rFVIIa may be administered. The only assays for plasma, PCC, or rFVIIa efficacy are TEG or TEM, which are not available everywhere, but the patient's recovery may be monitored indirectly using the PT/INR.

Autoanti-VIII Inhibitor and Acquired Hemophilia

Acquired autoantibodies that specifically inhibit factors II (prothrombin), V, VIII, IX, and XIII and VWF have been described in nonhemophilic patients.⁶⁴ Autoanti-VIII is the most common. Patients who develop an autoantibody to factor VIII, which is diagnostic of acquired hemophilia, are frequently older than age 60 and have no apparent underlying disease. Acquired hemophilia is occasionally associated with rheumatoid arthritis, inflammatory bowel disease, systemic lupus erythematosus, or lymphoproliferative disease. Pregnancy appears to trigger acquired hemophilia 2 to 5 months after delivery. Patients with inhibitor autoantibodies are prescribed immunosuppressive therapy, although autoantibodies that develop after pregnancy typically disappear spontaneously. Acquired hemophilia has an overall incidence of 1 per million people per year. Patients experience sudden and severe bleeding in soft tissues or bleeding in the gastrointestinal or genitourinary tract. Acquired hemophilia, even when treated, remains fatal in at least 20% of cases. Autoantibodies to other procoagulants are less frequent but create similar symptoms.⁶⁵

Clot-Based Assays in Acquired Hemophilia

Laboratory directors recommend the PT, PTT, and thrombin time for any patient with sudden onset of anatomic hemorrhage that resembles acquired hemophilia. In the presence of a factor VIII inhibitor, which is the most common inhibitor, the PTT is likely to be prolonged, whereas the lab practitioner expects the PT and thrombin time to be normal. A factor assay should reveal factor VIII activity to be less than 30% and often undetectable.

The laboratory professional confirms the presence of the inhibitor with clot-based mixing studies. The PTT prolongation may be corrected initially by the addition of normal plasma (NP) to the test specimen in a 1:1 ratio, but PTT again becomes

prolonged after incubation of the 1:1 NP-patient plasma mixture at 37° C for 1 to 2 hours. The return of prolongation after incubation occurs because factor VIII autoantibodies are frequently of the immunoglobulin G4 isotype, which are time and temperature dependent. Consequently, the inhibitor effect may be evident only after the patient's inhibitor is allowed to interact with the factor VIII in the NP for 1 to 2 hours at 37° C before testing. A few high-avidity inhibitors may cause immediate prolongation of the PTT; in this case an incubated mixing study is unnecessary.

The in vitro kinetics of factor VIII neutralization by an autoantibody are nonlinear. Although there is early rapid loss of factor VIII activity, residual activity remains, which indicates that the reaction has reached equilibrium. This is called *type II kinetics* (Figure 38-2). In contrast, alloantibodies to factor VIII, which develop in 20% to 25% of patients with severe hemophilia in response to factor VIII therapy, exhibit type I kinetics. In the latter, there is linear in vitro neutralization of factor VIII activity over 1 to 2 hours, which results in complete inactivation. In type I kinetics, in vitro measurement is relatively accurate, whereas in type II kinetics, the titration of inhibitor activity is semiquantitative.⁶⁶

Quantitation of autoanti-VIII inhibitor is accomplished using the *Bethesda assay*, which is ordinarily employed to measure inhibitors in hemophilic patients with alloantibodies to factor VIII (see Hemophilia A and Factor VIII Inhibitors). Titer results help the clinician choose the proper therapy to control bleeding. Repeat titers are used to follow the response to immunosuppressive drugs but are not needed for management of the bleeding symptoms.

Factor Inhibitors Other Than Autoanti-Factor VIII

Anticoagulation factor II (anti-prothrombin) antibodies, detectable by immunoassay, develop in approximately 30% of patients with lupus anticoagulant.⁶⁷ Although lupus anticoagulant is associated with thrombosis, some patients with antiprothrombin antibodies experience bleeding and have a prolonged PT. A finding of reduced activity on prothrombin assay and a positive test result for lupus anticoagulant confirm the diagnosis. Antiprothrombin antibodies not associated with lupus anticoagulant are rare.

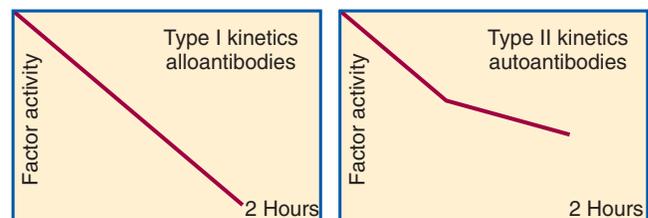


Figure 38-2 In type I linear kinetics, the inhibitor fully inactivates the factor in vitro. This is typical of alloantibodies such as factor VIII inhibitors in patients with severe hemophilia A. In type II kinetics, the inhibitor and factor reach equilibrium. This is typical of autoantibodies such as the factor VIII inhibitor in acquired hemophilia A. Because of the type II kinetics, the Bethesda titer is considered a semiquantitative measure in acquired hemophilia, although the results are commonly used to distinguish low-titer from high-titer antibodies.

Factor V and XIII inhibitors have been documented in patients receiving isoniazid treatment for tuberculosis.^{68,69} Antibodies to factor IIa (thrombin) and factor V may arise after exposure to topical bovine thrombin or fibrin glue.⁷⁰ Fibrin glue-generated autoantibodies have become less prevalent since 2008 when fibrin glue preparations began to use recombinant human thrombin instead of bovine thrombin. Autoanti-X antibodies are rare; however, factor X deficiency in amyloidosis may be caused by what seems to be an absorptive mechanism.⁷¹ In many cases of acquired inhibitors, mixing studies show uncorrected prolongation without incubation (immediate mixing study), and inhibitor titers may be determined by the Bethesda procedure.

Acquired Hemophilia Management

Activated PCC or rFVIIa may bypass the coagulation factor VIII inhibitor in acquired hemophilia and thereby control acute bleeding. Patients who have low titers of inhibitor (less than 5 Bethesda units) may respond to administration of desmopressin acetate (DDAVP) or factor VIII concentrates, but close monitoring of their response to therapy with serial coagulation factor VIII activity assays is warranted. Once bleeding is controlled, immunosuppressive therapy may reduce the inhibitor titer.⁷² Plasma exchange may also be used in severe cases, but the response is less reliable than the response to immunosuppression.

Acquired von Willebrand Disease

Acquired VWF deficiency, with symptoms similar to those of congenital VWD, has been described in association with hypothyroidism; autoimmune, lymphoproliferative, and myeloproliferative disorders; benign monoclonal gammopathies; Wilms tumor; intestinal angiodysplasia; congenital heart disease; pesticide exposure; and hemolytic uremic syndrome.⁷³ The pathogenesis of acquired VWD varies and may involve decreased production of VWF, presence of an autoantibody, or adsorption of VWF to abnormal cell surfaces, as seen in association with lymphoproliferative disorders and Wilms tumor.⁷⁴

Acquired VWD manifests with moderate to severe mucocutaneous bleeding and may be suspected in any patient with recent onset of bleeding who has no significant medical history. Although the PT is not affected, the PTT may be moderately prolonged if the VWF reduction is severe enough to cause a deficiency of coagulation factor VIII, for which VWF serves as a carrier molecule. As in congenital VWD, the diagnosis is based on a finding of diminished VWF activity (ristocetin cofactor [VWF:RCo] assay) and diminished VWF antigen (VWF:Ag) by immunoassay. It may be difficult to differentiate between mild, previously asymptomatic congenital VWD and acquired VWD.

If the patient requires treatment for bleeding, DDAVP or a plasma-derived factor VIII/VWF concentrate such as Humate-P® (CSL Behring, King of Prussia, PA), Wilate® (Octapharma, Hoboken, NJ), or Alphanate® (Grifols, Los Angeles, CA) is effective at controlling the symptoms. Cryoprecipitate is no longer recommended for treatment of VWD because it does not undergo viral inactivation.

Disseminated Intravascular Coagulation

DIC, although characteristically identified through its hemorrhagic symptoms, is classified as a thrombotic disorder and is described in Chapter 39.

CONGENITAL COAGULOPATHIES

Von Willebrand Disease

VWD is a common mucocutaneous bleeding disorder first described by Finnish professor Erik von Willebrand in 1926. VWD is caused by any one of dozens of germline mutations that result in quantitative or structural abnormalities of VWF. Both quantitative and structural abnormalities lead to decreased adhesion by platelets to injured vessel walls, causing impaired primary hemostasis. VWD is the most prevalent of the congenital bleeding disorders and is found in approximately 1% of the population. It affects both sexes because of its autosomal dominant inheritance pattern. The parameters of and laboratory testing guidelines for VWD are established and defined in the National Heart, Lung, and Blood Institute (NHLBI) publication *The Diagnosis, Evaluation, and Management of von Willebrand Disease*.⁷⁵

Molecular Biology and Functions of von Willebrand Factor

VWF is a glycoprotein whose molecular mass ranges from 800,000 to 20,000,000 Daltons, the largest molecule in human plasma. Its plasma concentration is 0.5 to 1.0 mg/dL, but a great deal more is readily available on demand from storage organelles. VWF is synthesized in the endoplasmic reticulum of endothelial cells and stored in cytoplasmic *Weibel-Palade bodies* of endothelial cells. It is also synthesized in megakaryocytes and stored in the α -granules of platelets (Chapter 13). Weibel-Palade bodies and α -granules release VWF in response to a variety of hemostatic stimuli.⁷⁶

The VWF gene consists of 52 exons spanning 178 kilobase pairs (kb) on chromosome 12.⁷⁷ The translated protein is a monomer of 2813 amino acids that, after glycosylation, forms dimers that are transferred to the aforementioned storage organelles, where the dimers polymerize to form enormous multimers. At the time of storage, a signal sequence and a propeptide, known as *VWF antigen II*, are cleaved so that the mature monomers, already polymerized, consist of 2050 amino acids.⁷⁸

As described in Chapter 37, VWF-cleaving protease (ADAMTS-13) cleaves the ultra-large VWF multimers of the Weibel-Palade bodies at their moment of release into monomers of various size. ADAMTS-13 deficiency allows for release of the ultra-large multimers, the basis for the devastating disorder, thrombotic thrombocytopenic purpura (Chapter 40)

Each VWF monomer has four functional domains that bind factor VIII, platelet glycoprotein Ib/IX/V, platelet glycoprotein IIb/IIIa, and collagen (Chapter 13). Upon release from intracellular stores, VWF forms a complex with coagulation factor VIII. This complex is named *VIII/VWF* (Figure 38-3). VWF protects factor VIII from proteolysis, prolonging its plasma half-life from a few minutes without VWF to 8 to 12 hours with

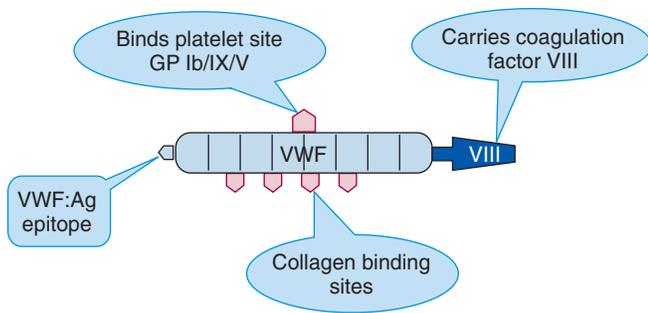


Figure 38-3 Large von Willebrand factor (VWF) multimers bind intimal collagen upon desquamation of endothelial cells or more extensive injury and expand under shear force to form a “carpet” to which platelets adhere through platelet glycoprotein (GP) Ib/IX/V binding sites. VWF binds and stabilizes factor VIII, serving as a carrier protein. The VWF antigen (VWF:Ag) is the target of quantitative immunoassays.

VWF. **Table 38-3** lists the nomenclature entries for the structural and functional components of the factor VIII/VWF molecule.

Although VWF is the factor VIII carrier molecule, its primary function is to mediate platelet adhesion to subendothelial collagen in areas of high flow rate and high shear force, as in capillaries and arterioles. VWF first binds fibrillar intimal collagen exposed during the desquamation of endothelial cells. Subsequently, platelets adhere through their glycoprotein Ib/IX/V receptor to the VWF “carpet.” The largest VWF multimers are

TABLE 38-3 Nomenclature Relating to the Factor VIII/von Willebrand Factor Complex

Term	Meaning
VIII/VWF	Customary term for the plasma combination of factor VIII and VWF.
VIII	Procoagulant factor VIII, the protein transported on VWF. Factor VIII binds activated factor IX to form the complex of VIIIa-IXa, which digests and activates factor X. Factor VIII deficiency is called <i>hemophilia A</i> .
VWF:Ag	Epitope that is the antigenic target for the VWF immunoassay.
VWF:RCo	Ristocetin cofactor activity, also called <i>VWF activity</i> ; VWF activity is measured by the ability of ristocetin to cause agglutination of reagent platelets by the patient's VWF.
VWF:CBA	Collagen binding assay, a second method for assaying VWF activity. Large VWF multimers bind immobilized target collagen, predominantly collagen III.
VWF:Immunoactivity	Automated nephelometric activity assay that employs latex microparticles and monoclonal anti-glycoprotein I-VWF receptor, a third method for assaying VWF activity.
VIII:C	Factor VIII coagulant activity as measured in factor-specific clot-based assays.

best equipped to serve the adhesion function. When VWF binds glycoprotein Ib/IX/V, platelets become activated and express a second VWF binding site, glycoprotein IIb/IIIa. This receptor binds VWF and fibrinogen to mediate irreversible platelet-to-platelet aggregation. The adhesion and aggregation sequences are essential to normal hemostasis.

Pathophysiology of von Willebrand Disease

Structural (qualitative) or quantitative VWF abnormalities reduce platelet adhesion, which leads to mucocutaneous hemorrhage of varying severity: epistaxis, ecchymosis, menorrhagia, gastrointestinal tract bleeding, hematemesis, and surgical bleeding. Symptoms vary over time and within kindreds because VWF production and release are susceptible to a variety of physiologic influences. Severe quantitative VWF deficiency creates in addition factor VIII deficiency owing to the inability to protect nonbound factor VIII from proteolysis. Many people have VWF levels in the intermediate range of 30% to 50% of normal and maintain a factor VIII level sufficient for competent coagulation.⁷⁹ When factor VIII levels decrease to less than 30% of normal, anatomic soft tissue bleeding accompanies the typical mucocutaneous bleeding pattern of VWD.

Von Willebrand Disease Types and Subtypes

Type 1 von Willebrand Disease. Type 1 VWD is a quantitative VWF deficiency caused by one of several autosomal dominant frameshifts, nonsense mutations, or deletions.^{80,81} Type 1 is seen in approximately 75% of VWD patients. The plasma concentrations of all VWF multimers and factor VIII are variably, although proportionally, reduced (**Figure 38-4**).⁸² There is mild to moderate bleeding, usually following a hemostatic challenge such as dental extraction or surgery. In women, menorrhagia is a common complaint that leads to the diagnosis of VWD.

Type 2 von Willebrand Disease. Type 2 VWD comprises a variety of qualitative VWF abnormalities. VWF levels

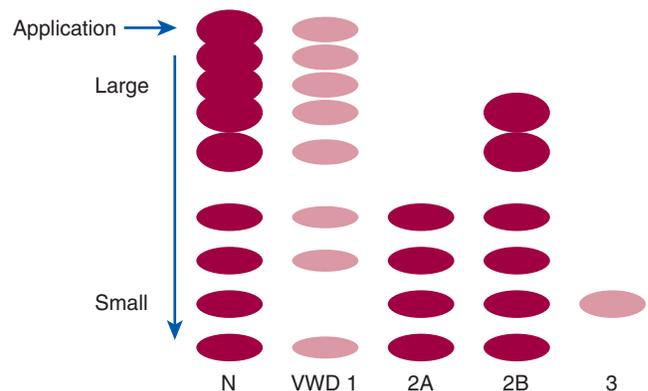


Figure 38-4 Schematic of von Willebrand factor multimer analysis by polyacrylamide gel electrophoresis shows diminished concentration but normal ratios in von Willebrand disease (VWD) type 1, absence of large and intermediate multimers in subtype 2A, absence of large forms in subtype 2B, and absence of all multimers in type 3.

may be normal or moderately decreased, but VWF function is consistently reduced.

Subtype 2A von Willebrand Disease. Ten percent to 20% of VWD patients have subtype 2A, which arises from well-characterized autosomal dominant point mutations in the A2 structural domain of the VWF molecule. These mutations render VWF more susceptible to proteolysis by ADAMTS-13, which leads to a predominance of small-molecular-weight multimers in the plasma (Figure 38-4). The smaller multimers support less platelet adhesion activity than the normal high- or intermediate-molecular-weight multimers. Patients with subtype 2A VWD have normal or slightly reduced VWF:Ag levels, with markedly reduced VWF activity as a result of the loss of the high-molecular-weight and intermediate-molecular-weight multimers essential for platelet adhesion.⁸³

Subtype 2B von Willebrand Disease. In subtype 2B VWD, rare mutations within the A1 domain raise the affinity of VWF for platelet glycoprotein Ib/IX/V, its customary binding site; these are thus “gain-of-function” mutations. Large VWF multimers spontaneously bind resting platelets and are unavailable for normal platelet adhesion. Consequently, the electrophoretic multimer pattern is characterized by lack of high-molecular-weight multimers but presence of intermediate-molecular-weight multimers (Figure 38-4). There also may be moderate thrombocytopenia caused by chronic platelet activation because multimer-coated platelets indiscriminately bind the endothelium.

A platelet mutation that increases glycoprotein Ib/IX/V affinity for normal VWF multimers creates a clinically similar disorder called *platelet-type VWD* or *pseudo-VWD*. In this instance, the large multimers also are lost from the plasma, and platelets become adhesive. Both clinically and in the laboratory, the two entities are indistinguishable.

Subtype 2M von Willebrand Disease. Subtype 2M VWD describes a qualitative variant of VWF that has decreased platelet receptor binding but a normal multimeric pattern in electrophoresis. The distinguishing feature of subtype 2M that separates it from type 1 is a discrepancy between the concentration of VWF:Ag and its activity as measured using the VWF:RCo assay.

Subtype 2N von Willebrand Disease (Normandy Variant; Autosomal Hemophilia). A rare autosomal VWF missense mutation impairs its factor VIII binding site. This condition results in factor VIII deficiency despite normal VWF:Ag concentration and activity and a normal multimeric pattern. The disorder is also known as *autosomal hemophilia* because its clinical symptoms are indistinguishable from the symptoms of hemophilia except that it affects both males and females. Subtype 2N is suspected when a girl or woman is diagnosed with hemophilia after soft tissue bleeding symptoms. In boys or men, subtype 2N is suspected when a patient misdiagnosed with hemophilia A fails to respond to factor VIII concentrate therapy. The poor response occurs because the factor has a plasma

half-life of mere minutes when it cannot be bound by VWF. The diagnosis of VWD subtype 2N is confirmed using a molecular assay that detects the specific mutation responsible for the abnormal factor VIII binding to VWF.

Type 3 von Willebrand Disease. Autosomal recessive VWF gene translation or deletion mutations produce severe mucocutaneous and anatomic hemorrhage in compound heterozygotes or, in consanguinity, homozygotes. In this rare disorder, VWF is absent or nearly absent from plasma (Figure 38-4). Factor VIII is also proportionally diminished or absent, and primary and secondary hemostasis is impaired.

Laboratory Detection and Classification of von Willebrand Disease

Definitive diagnosis of VWD depends on the combination of a personal and family history of mucocutaneous bleeding and the laboratory demonstration of decreased VWF activity. A CBC is necessary to rule out thrombocytopenia as the cause of mucocutaneous bleeding, and PT and PTT, which assess the coagulation system, are part of the initial VWD workup. No longer recommended, according to the 2009 NHLBI VWD guidelines, are the *bleeding time* test and the PFA-100 or other automated functional platelet assays (Chapters 42 and 44). These traditional screening tests generate “conflicting” sensitivity and specificity data.⁸⁴

The standard VWD test panel includes three assays: a *quantitative VWF* test (VWF:Ag assay) employing enzyme immunoassay or automated latex immunoassay methodology, such as the Liatest (Diagnostica Stago, Asnières-sur-Seine, France); the *VWF activity* test, which determines the factor’s ability to bind to platelets, also known as the *VWF:RCo assay*; and a factor VIII activity assay. The VWF:RCo assay employs preserved reagent platelets. The agonist ristocetin supports platelet agglutination in the presence of VWF.

When the ratio of the VWF:RCo assay value to the VWF:Ag concentration is less than 0.5, 0.6, or 0.7 (ratio cutoff is generated from reference interval studies by local laboratories), the laboratory professional infers qualitative or type 2 VWD. Additional tests are needed to confirm type 2 VWD and to differentiate subtype 2A from subtype 2B. *Low-dose ristocetin-induced platelet aggregometry* (RIPA), also called the *ristocetin response curve*, identifies subtype 2B. The low-dose RIPA test is performed on platelet-rich plasma. In subtype 2B the patient’s platelets, because they are coated with abnormal VWF multimers, agglutinate in response to less than 0.5 mg/mL ristocetin; sometimes they even agglutinate in response to 0.1 mg/mL ristocetin. In comparison, normal platelets or platelets from a patient with subtype 2A agglutinate only at ristocetin concentrations greater than 0.5 mg/mL.

VWF multimer analysis by sodium dodecyl sulfate–polyacrylamide gel electrophoresis further differentiates between VWD subtypes 2A and 2B. Although both 2A and 2B lack high-molecular-weight multimers, intermediate multimers are present in the electrophoretic pattern of subtype 2B VWD samples but are absent from the pattern of a patient with subtype 2A VWD. Multimer analysis is technically demanding and is performed

mainly in reference laboratories for differentiation of VWD subtypes. Table 38-4 lists the expected test results for all VWD types and subtypes.

Because acquired VWD may mimic any one of the VWD types and subtypes, results of the laboratory workup do not help to differentiate it from the inherited condition. Review of the clinical history with emphasis on age at onset of bleeding, comorbid conditions, and family history may signal acquired disease rather than the congenital form.

Pitfalls in von Willebrand Disease Diagnosis

Varying genetic penetrance, ABO blood group, inflammation, hormones, age, and physical stress influence VWF activity.⁸⁵ Increased estrogen levels during the second and third trimesters of pregnancy nearly normalize plasma VWF activity even in women with moderate VWF deficiency. However, VWF function and concentration decrease rapidly after delivery, which may lead to acute postpartum hemorrhage, for which the obstetrician is watchful. Oral contraceptive use and hormone replacement therapy also raise VWF activity, and activity waxes and wanes with the menstrual cycle.

VWF activity rises substantially in acute inflammation such as occurs postoperatively, subsequent to trauma, or during an infection. Physical stress such as cold, exertion, or children's crying or struggling during venipuncture causes VWF activity to rise. VWF activity rises when the phlebotomist allows the tourniquet to remain tied for more than 1 minute prior to venipuncture and drops if the specimen is stored in the refrigerator prior to testing. VWD patients experience fluctuation in disease severity over time, and the clinical manifestations of the disease vary from person to person within kindred, despite the assumption that everyone in the family possesses the same mutation. When the clinical presentation suggests VWD, the VWD laboratory assay panel should be repeated until the results are conclusive.⁸⁶

Adding to VWD diagnostic confusion is concern over the variability in the results of the VWF:RCo assay, which is based on platelet aggregometry but is performed using a variety of instruments and methods, including at least one automated

device, the BCS XP System[®] with von Willebrand reagent (Siemens Healthcare Diagnostics, Inc., Deerfield, IL). Ristocetin avidity varies from lot to lot. In the United States, proficiency surveys consistently reveal VWF:RCo assays to have an unimpressive interlaboratory coefficient of variation of 30% and a least detectable activity range of 6% to 12%.⁸⁷

Concern over the poor reproducibility of results of the VWF activity assay has led to attempts at developing alternative assays, the most promising of which is the VWF collagen binding (VWF:CB) assay. VWF:CB employs type III collagen as its solid-phase target antigen. Developed in 1990, the VWF:CB assay produces results that more closely match those of the VWF:RCo assay than of VWF:Ag assays, because collagen type III binds predominantly large VWF multimers; however, the target collagen composition requires standardization before this assay achieves standard assay status.⁸⁸ The VWF:CB assay has better precision than the VWF:RCo assay.⁸⁹

Many reference laboratories add the VWF:CB assay to their initial VWD screening profile. They argue that the VWF:CB assay detects abnormalities of VWF collagen binding, whereas the VWF:RCo assay detects abnormalities of VWF platelet binding and cite instances in which the VWF:CB value was abnormal when the VWF:RCo value was normal, and vice versa.⁹⁰

A second alternative to VWF:RCo is the automated HemosIL von Willebrand Factor Activity assay (Instrumentation Laboratory Company [IL], Bedford, MA). This nephelometric immunoassay employs latex microparticles coated with monoclonal antibodies directed to the VWF glycoprotein I binding site. Results compare well with the cofactor assay when the immunoassay is performed on IL instrumentation.⁹¹

In an effort to more closely reflect clinical reality and reduce false-positive type 1 VWD diagnoses, the 2009 NHLBI VWD guidelines have coined the nondisease description "low VWF" for the condition in which VWF activity and antigen concentrations are between 30% and 50% of normal, the ratio of VWF:RCo to VWF:Ag is greater than 0.5, and factor VIII activity is greater than 50% of normal. This suggested category reflects the difficulty in distinguishing mucocutaneous bleeding from self-reported "easy bruising" and recognizes that low VWF

TABLE 38-4 Laboratory Detection and Classification of von Willebrand Disease: Typical Results*

Laboratory Test	Type 1	Type 2A	Type 2B	Type 3
VWF activity via ristocetin cofactor, collagen binding, or immunoactivity assay	Low	Low	Low	Very low or absent
VWF antigen	Low	Normal to slightly decreased	Normal to slightly decreased	Very low or absent
VWF activity to VWF antigen ratio	>0.5	<0.5	<0.5	N/A
Platelet count	Normal	Normal	Decreased	Normal
Partial thromboplastin time (PTT)	Normal to slightly prolonged	Normal	Normal	Prolonged
RIPA	Decreased	Decreased	Increased	Absent
Factor VIII activity	Mildly low	Normal	Normal	<5%
VWF multimers	Normal pattern	Large and intermediate forms absent	Large forms absent	All forms absent

*Expected results are given, but results vary over time and within affected kindred.

N/A, Not applicable; RIPA, ristocetin-induced platelet aggregometry with 1 mg/mL ristocetin; VWF, von Willebrand factor.

activity and bleeding often associate coincidentally. To make a definite type 1 VWD diagnosis, 30% of normal VWF activity is used as the cutoff. Molecular confirmation of type 1 VWD, currently but a dream because of the plethora of contributing mutations, may soon become routine as molecular microchip technology meets the challenge.

VWF activity varies by ABO blood group, and expert panels have in the past recommended that laboratory directors maintain separate reference intervals for each group, as provided in Table 38-5. The 2009 NHLBI VWD guidelines have recommended against this practice, noting that “despite the ABO blood grouping and associated reference ranges, the major determinant of bleeding risk is low VWF.” Therefore, VWF test result reference intervals are now population based rather than ABO stratified. The internationally generalized cutoff of 50% for low VWF and 30% for VWD is clinically reasonable, although laboratory directors may choose to validate and adjust this cutoff in internal reference interval studies.

In a well-managed tertiary care facility, laboratory professionals and physicians communicate regularly with medical staff who are challenged with VWD management—for example, nurses, pharmacists, emergency department and primary care physicians, internists, surgeons, gynecologists, and hematologists. Laboratory professionals ensure that those who manage VWD patients are aware of the effects of ABO group, estrogen levels, inflammation, and physical stress on VWF activity and that they understand the strengths and limitations of VWF laboratory assay panels, the proper interpretation of assay results, and the availability of follow-up and confirmatory assays.

Treatment of von Willebrand Disease

Mild bleeding may resolve with the use of localized measures, such as limb elevation, pressure, and application of ice packs (the athlete’s acronym is *RICE* for rest, ice, compression, and elevation).⁹² Moderate bleeding may respond to *estrogen* and *desmopressin acetate*, which trigger the release of VWF from storage organelles. Therapeutic dosages are monitored when necessary using serial VWF:Ag concentration assays. Desmopressin acetate (1-desamino-8-D arginine vasopressin) is an antidiuretic hormone analogue used to control incontinence in diabetes mellitus and bed-wetting; release of VWF from storage organelles is a side effect. Desmopressin acetate in its oral form, DDAVP, or nasal spray form, Stimate (both from

CSL Behring, King of Prussia, PA), is consistently effective for type 1 VWD and generally useful for subtype 2A. It is contraindicated for subtype 2B, however, because it causes the release of abnormal VWF with increased affinity for platelet receptors, which may intensify thrombocytopenia and lead to increased platelet activation and consumption. Because of its antidiuretic property, repeated doses may lead to hyponatremia (low serum sodium). For this reason, it is necessary to monitor and regulate electrolytes during desmopressin acetate therapy.

The lysine analogues ϵ -aminocaproic acid (EACA; Amicar) and TXA (Cyklokapron) inhibit fibrinolysis and may help control bleeding when used alone or in conjunction with desmopressin acetate. Therapy using nonbiological preparations is preferred over human plasma-derived biologic therapy because nonbiologicals eliminate the risk of viral disease transmission and circumvent religious objections to receipt of human blood products.

For treatment of severe VWD (type 3) and subtype 2B, three commercially prepared human plasma-derived high-purity preparations are available that provide a mixture of VWF and coagulation factor VIII: Humate-P, Alphanate, and Wilate (Wilate was FDA-cleared in December 2009).⁹² The calculation of the proper dosage follows principles identical to those used for treatment of hemophilia A provided in the next section. Laboratory monitoring by the VWF:Ag assay is essential to determine if the given amount produced the target level of VWF and to follow its degradation between doses.

Recombinant and affinity-purified factor VIII preparations contain no VWF and cannot be used to treat VWD. Cryoprecipitate and plasma are less desirable alternatives because of the risk of virus transmission, and the necessary plasma volume per dose may cause TACO. Therapy for bleeding secondary to acquired VWD follows the same principles as delineated previously, plus treatment of the primary disease, if applicable.⁹³ Therapeutic recommendations for VWD are summarized in Table 38-6.

Hemophilia A (Factor VIII Deficiency)

The hemophilias are congenital single-factor deficiencies marked by anatomic soft tissue bleeding. Second to VWD in prevalence among congenital bleeding disorders, hemophilias occur in 1 in 10,000 individuals. Of those affected, 85% are deficient in factor VIII, 14% are deficient in factor IX, and 1%

TABLE 38-5 Von Willebrand Factor (VWF) Reference Intervals by Blood Group

Blood Group	Reference Interval
O	36%–157%
A	48%–234%
B	57%–241%
AB	64%–238%
Population based: “Low VWF”	<50%
Population based: von Willebrand disease	<30%

TABLE 38-6 Therapeutic Strategies in von Willebrand Disease

Type	Primary Approach	Other Options
1	Estrogen, DDAVP, EACA or TXA	Factor VIII/VWF concentrate
2A	Estrogen, DDAVP, EACA or TXA	Factor VIII/VWF concentrate
2B	Factor VIII/VWF concentrate	EACA
2N	Factor VIII/VWF concentrate	EACA
3	Factor VIII/VWF concentrate	Platelet transfusions

DDAVP, Desmopressin acetate; EACA, ϵ -aminocaproic acid; TXA, tranexamic acid; VWF, von Willebrand factor.

are deficient in factor XI or one of the other coagulation factors, such as factor II (prothrombin), V, VII, X, or XIII. Congenital deficiency of factor VIII is called *classic hemophilia* or *hemophilia A*.⁹⁴

Factor VIII Structure and Function

Factor VIII is a two-chain, 285,000 Dalton protein translated from the X chromosome.⁹⁵ When the coagulation cascade is activated, thrombin cleaves plasma factor VIII and releases a large polypeptide called the *B domain* that dissociates from the molecule. This leaves behind a calcium-dependent heterodimer that detaches from its VWF carrier molecule to bind phospholipid and factor IXa. The VIIIa/IXa complex, sometimes called *tenase complex*, cleaves and activates coagulation factor X at a rate 10,000 times faster than free IXa can cleave factor X. Consequently, factor VIII deficiency significantly slows the coagulation pathway's production of thrombin and leads to hemorrhage. In vitro, factor VIII (like factor V) is labile and deteriorates at about 5% an hour at room temperature.

Hemophilia A Genetics

The gene for factor VIII spans 186 kb of the X chromosome and is the site of various deletions, stop codons, and nonsense and missense mutations. Most of these mutations result in quantitative disorders in which the factor VIII coagulant activity and antigen concentration match, but in rare cases low activity is seen despite normal antigen concentration.⁹⁶ The latter cases are due to qualitative or structural factor VIII abnormalities traditionally known as *cross-reacting material positive* disorders.⁹⁷

Male hemizygotes, whose sole X chromosome contains a factor VIII gene mutation, experience anatomic bleeding, but female heterozygotes, who are carriers, do not.⁹⁸ When a female carrier has children with an unaffected man, the chances of hemophilia A inheritance are 25% chance of a normal daughter, 25% chance of a carrier daughter, 25% chance of a normal son, and 25% chance of a hemophilic son. All sons of men with hemophilia A and noncarrier women are normal, whereas all daughters are obligate carriers of the disease.⁹⁹ In addition, approximately 30% of newly diagnosed cases arise as a result of spontaneous germline mutations in individuals who have no family history of hemophilia A.¹⁰⁰ Rarely, the symptoms of hemophilia A may be seen in females. This phenomenon could be due to true homozygosity or double heterozygosity, such as in the female offspring of a hemophilic father and a carrier mother. Other possibilities include a spontaneous germline mutation in the otherwise normal allele of a heterozygous female or a disproportional inactivation of the X chromosome with the normal gene, termed *extreme lyonization*. Finally, VWD of the Normandy subtype may present as mild hemophilia A in males and females.

Hemophilia A Clinical Manifestations

Hemophilia A causes anatomic bleeds with deep muscle and joint hemorrhages; hematomas; wound oozing after trauma or surgery; and bleeding into the central nervous system, peritoneum,

gastrointestinal tract, and kidneys. Acute joint bleeds (hemarthroses) are exquisitely painful and cause temporary immobilization. Chronic joint bleeds cause inflammation and eventual permanent loss of mobility, whereas bleeding into muscles may cause nerve compression injury, with first temporary and then lasting disability. Cranial bleeds lead to severe, debilitating, and durable neurologic symptoms, such as loss of memory, paralysis, seizures, and coma, and may be rapidly fatal. Bleeding may begin immediately after a triggering event or may become manifest after a delay of several hours. Some bleeding seems to be spontaneous.

The diagnosis of hemophilia A begins with laboratory testing after the birth of an infant to a mother who has a family history of hemophilia. In the absence of a family history, abnormal bleeding in the neonatal period, which may appear as easy bruising, bleeding from the umbilical stump, postcircumcision bleeding, hematuria, or intracranial bleeding, is considered suspicious for hemophilia. Severe hemophilia usually is diagnosed in the first year of life, whereas mild hemophilia may not become apparent until a triggering event such as trauma, surgery, or dental extraction occurs in late childhood, adolescence, or adulthood.

The laboratory diagnosis of coagulopathies in the newborn or older infant is complicated by the requirement for an unheparinized specimen of at least 2 mL in volume from tiny veins and by the typically low newborn levels of some coagulation factors. Medical laboratory practitioners expect the PT and PTT to be prolonged because of physiologically low levels of factors II (prothrombin), VII, IX, and X, even in full-term infants. Factor VIII levels are similar to the levels in adults, even in infants who are born prematurely; this allows skillful laboratory staff to provide the correct diagnosis of hemophilia A using a factor VIII activity assay.

The severity of hemophilia A symptoms is inversely proportional to factor VIII activity. Laboratory professionals classify an activity level of less than 1% as severe, associated with spontaneous or exaggerated bleeding in the neonatal period. Activity levels of 1% to 5% are seen in moderate hemophilia, which is usually diagnosed in early childhood after symptoms become apparent. In mild hemophilia, with activity levels of 5% to 30%, hemorrhage follows significant trauma and becomes a risk factor mainly in surgery or dental extractions, and patients may go for long periods without symptoms.

Hemophilia A Complications

As a result of frequent bleeds, hemophilia patients often have debilitating and progressive musculoskeletal lesions and deformities and neurologic deficiencies after intracranial hemorrhage. In addition, other effects of chronic diseases, such as limited productivity, low self-esteem, poverty, drug dependency, and depression, are common problems. Before the advent of sterilized and recombinant factor concentrates, chronic hepatitis often resulted from repeated exposure to blood products. Tragically, 70% of hemophiliacs treated before 1984 are human immunodeficiency virus (HIV) positive or have died from acquired immunodeficiency syndrome.¹⁰¹

Hemophilia A Laboratory Diagnosis

The laboratory workup for a suspected congenital single coagulation factor deficiency starts with the PT, PTT, and thrombin time and continues with factor assays based on the results of the screening tests. Before the physician initiates laboratory testing, however, he or she records a history of the patient's hemorrhagic symptoms. In hemophilia A, the PT and thrombin time are likely to be normal, and the PTT is prolonged, provided that the PTT reagent is sensitive to factor VIII deficiencies at or less than the 30% plasma level. Table 38-7 lists the expected results for each clot-based screening assay for any single-factor deficiency associated with bleeding, including deficiencies of fibrinogen and factors II (prothrombin), V, VII, VIII, IX, X, and XI. Deficiencies of contact factors (factor XII, high-molecular-weight kininogen, and prekallikrein) have no relationship to bleeding, and these factors do not appear in Table 38-7.

Hemophilia A Carrier Detection

Approximately 90% of female carriers of hemophilia A are detected by measuring the ratio of factor VIII activity to VWF:Ag value (VIII:VWF). This ratio is effective because VWF production is unaffected by factor VIII deficiency. Using a ratio, rather than a factor VIII assay value alone, normalizes for some of the physiologic variables that affect factor VIII activity and VWF:Ag assays, such as estrogen levels, inflammation, stress, and exercise.

The laboratory professional establishes a reference interval for the VIII:VWF ratio using plasmas from at least 30 women who do not have factor VIII deficiency. If the ratio of the individual being tested is below the lower limit of the interval, she is likely to be a carrier. These results may be influenced by excessive lyonization, variation in VWF production, and analytical variables; consequently, if carrier status is suspected and the VIII:VWF ratio is greater than the lower limit of the reference range, genetic testing may be necessary to detect one of the many polymorphisms associated with factor VIII deficiency.

Hemophilia A Treatment

The goal of on-demand hemophilia A treatment is to raise the patient's factor VIII activity to hemostatic levels whenever he or

she experiences or suspects a bleeding episode or anticipates a hemostatic challenge such as a surgical procedure. The target activity level depends on the nature of the bleeding and the procedure, but it is seldom necessary to reach activity greater than 75%. Target activity should be maintained until the threat is resolved. In the case of a bleed into soft tissue or a body cavity, the sooner the target factor level is reached, the less painful the episode, and the less likely the patient is to experience inflammation or nerve damage. Because factor VIII has a half-life of 8 to 12 hours, twice-a-day infusions are required.

With the availability of abundant recombinant factor VIII concentrate, many hemophilic patients maintain themselves on a steady prophylactic dosage designed to constantly keep their factor VIII activity at hemostatic levels.¹⁰² Although it is initially more expensive, the prophylactic approach conserves downstream resources by ameliorating the adverse effects of repeated hemorrhages and their long-term consequences.¹⁰³

Many hemophilic patients' factor VIII activity rises upon administration of desmopressin acetate in the form of DDAVP or Stimate (nasal formulation), alone or in combination with an antifibrinolytic such as EACA or TXA. When desmopressin acetate treatment proves ineffective, intravenous factor VIII concentrates are the next option. High-purity factor VIII concentrates are produced from mammalian cells using recombinant DNA technology or are derived from human plasma using factor VIII-specific monoclonal antibodies and column separation.^{104,105} The human plasma-derived concentrates Alphanate, Humate-P, and Wilate are prepared by chemical fractionation of human plasma and contain VWF, fibrinogen, and noncoagulant proteins, in addition to factor VIII. All plasma-derived concentrates undergo viral inactivation steps, and since 1985, none has transmitted lipid-envelope viruses such as HIV, hepatitis B virus, or hepatitis C virus. Plasma-derived factor VIII concentrates, however, may transmit nonlipid viruses such as parvovirus B19 and hepatitis A virus.¹⁰⁶ Recombinant products may use human albumin in the manufacturing process, which introduces the theoretical risk of Creutzfeldt-Jakob disease transmission; however, manufacturers now provide products free of all human protein, such as Bioclote (Pfizer, New York, NY).¹⁰⁷

TABLE 38-7 Results of Clot-Based Screening Assays in Congenital Single-Factor Deficiencies*

Deficient Factor	PT	PTT	TCT	Reflex Test
Fibrinogen	Prolonged	Prolonged	Prolonged	Fibrinogen assay
Prothrombin	Prolonged	Prolonged	Normal	Prothrombin, V, VII, and X assays
V	Prolonged	Prolonged	Normal	Prothrombin, V, VII, and X assays
VII	Prolonged	Normal	Normal	VII assay
VIII	Normal	Prolonged	Normal	VIII, IX, and XI assays
IX	Normal	Prolonged	Normal	VIII, IX, and XI assays
X	Prolonged	Prolonged	Normal	Prothrombin, V, VII, and X assays
XI	Normal	Prolonged	Normal	VIII, IX, and XI assays
XIII	Normal	Normal	Normal	Factor XIII quantitative assay

PT, Prothrombin time; PTT, partial thromboplastin time; TCT, thrombin clotting time.

*Results are valid when no anticoagulants are in use. Results may vary in response to reagent sensitivities.

When hematologists treat a hemophilic patient, they base their factor VIII concentrate dosage calculations on the definition of a unit of factor VIII activity, which is the mean amount present in 1 mL of normal plasma and is synonymous with 100% activity. They further calculate the desired increase after factor VIII concentrate infusion by subtracting the patient's preinfusion factor activity from the target activity level. The desired increase is multiplied by the patient's plasma volume to compute the dosage. The patient's plasma volume may be estimated from blood volume and hematocrit.¹⁰⁸ The blood volume is approximately 65 mL/kg of body weight, and the plasma volume is the plasmacrit (100% – hematocrit %) × blood volume, as in the following formulas:

$$\begin{aligned} \text{Plasma volume} &= \text{weight in kilograms} \times 65 \text{ mL/kg} \times \\ &\quad (1 - \text{hematocrit}) \\ \text{Factor VIII concentrate dose} &= \text{plasma volume} \times \\ &\quad (\text{target factor VIII level} - \text{initial factor VIII level}) \end{aligned}$$

This formula is also used in the treatment of VWD. Overdosing seems to confer no thrombotic risk, but it wastes resources. Regardless of the dose administered, laboratory monitoring and close clinical observation are essential to prevent or halt bleeding and its complications. Repeat dosing is done on an 8- to 12-hour schedule reflecting the half-life of factor VIII. The second administration of factor VIII uses half the concentration of the first dose.

Hemophilia A and Factor VIII Inhibitors

Alloantibody inhibitors of factor VIII arise in response to treatment in 30% of patients with severe hemophilia and 3% of those with moderate hemophilia. The laboratory practitioner suspects the presence of an inhibitor when bleeding persists or when the plasma factor VIII activity fails to rise to the target level after appropriate concentrate administration. Most factor VIII inhibitors are immunoglobulin G4, non-complement-fixing, warm-reacting antibodies. It is impossible to predict which patients are likely to develop inhibitors based on genetics, demographics, or the type of concentrate used.¹⁰⁹

The first step in inhibitor detection is a factor VIII assay. If the factor VIII activity exceeds 30%, no inhibitor is present. If the level is less than 30%, the laboratory practitioner proceeds to mixing studies. When the test plasma from the bleeding patient has a prolonged PTT, it is mixed 1:1 with normal plasma (NP), it is incubated 1 to 2 hours at 37° C, and the PTT of the mixture is measured. If no inhibitor is present, the incubated mixture should produce a PTT result within 10% of the incubated NP PTT. If an inhibitor is present, however, the factor VIII from the normal plasma is partially neutralized, and the mixture's PTT remains prolonged or "uncorrected," presumptive evidence of the inhibitor.

If mixing studies and the therapeutic results suggest the presence of a factor VIII inhibitor, a *Bethesda assay* is used to quantitate the inhibitor. NP with 100% factor activity is mixed at increasing dilutions in a series of tubes with the full-strength patient plasma. Factor VIII assays are performed on

each mixture. The operator then compares the results of the various dilutions and expresses the titer as Bethesda units. One Bethesda unit is the reciprocal of the dilution that caused neutralization of 50% of the factor VIII from the NP. The same assay is employed to measure factor VIII inhibitors in acquired hemophilia. Although the complex kinetics of acquired autoantibodies diminishes the accuracy of the results in acquired hemophilia, this method is adequate to monitor therapy.

Hemophilia patients with inhibitors are classified as *low* or *high responders*. Low responders generate inhibitor titers of 5 Bethesda units or lower and their inhibitor titers do not increase significantly following factor VIII administration. High responders generate inhibitor titers that exceed 5 Bethesda units and their antibody titers further rise in response to therapy. Low responders may be managed with raised factor VIII doses alone, whereas high responders may require activated prothrombin complex concentrate such as FEIBA (factor eight inhibitor bypassing activity) to control bleeding plus steroid or immunomodulation therapy to reduce the inhibitor antibody titer. Each laboratory director may choose to maintain a database of hemophilia patients who have inhibitors because previous titers often predict future inhibitor behavior.

Hemophilia A Treatment in Patients with Inhibitors

Every hemophilic patient with an inhibitor needs an individualized treatment plan to control bleeding episodes. Low responders often experience cessation of bleeding upon administration of large doses of factor VIII concentrate and may be so maintained. High responders may gain no benefit from factor VIII concentrates and instead are treated with activated PCC, Autoplex T or FEIBA, which generates thrombin in the presence of factor VIII inhibitors. The activated PCC dosage should not exceed 200 units/kg per day, distributed in two to four injections, because the activated factors may trigger DIC. NovoSeven also bypasses the physiologic factor VIII requirement, because it promotes thrombin formation through the tissue factor pathway.¹¹⁰ The discussions in this chapter of ACOTS and acquired hemophilia provide additional detail on activated PCC dosages.

Hemophilia B (Factor IX Deficiency)

Hemophilia B, also called *Christmas disease*, totals approximately 14% of hemophilia cases in the United States, although its incidence in India nearly equals that of hemophilia A.⁹⁴ Hemophilia B is caused by deficiency of factor IX, one of the vitamin K–dependent serine proteases. Factor IX is a substrate for both factors XIa and VIIa because it is cleaved by either to form dimeric factor IXa (Figure 38-5). Subsequently, factor IXa complexes with factor VIIIa to cleave and activate its substrate, factor X. Factor IX deficiency reduces thrombin production and causes soft tissue bleeding that is indistinguishable from that in hemophilia A. It also is a sex-linked, markedly heterogeneous disorder involving numerous separate mutations resulting in a range of mild to severe bleeding manifestations. Determination of female carrier status is less successful in

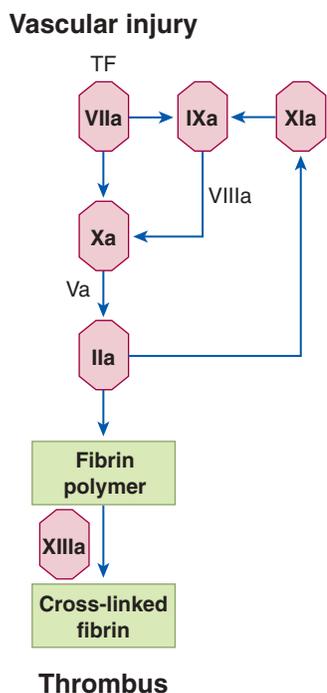


Figure 38-5 Simplified coagulation cascade mechanism showing positions of all factors whose absence may cause hemorrhage. *TF*, Tissue factor.

hemophilia B than in hemophilia A because of the large number of factor IX mutations and the lack of a linked molecule such as VWF that can be used as a normalization index. DNA analysis occasionally may be used to establish carrier status when hemophilia B has been diagnosed and its specific mutation identified in a relative.

The laboratory is essential to the diagnosis of hemophilia B. The PTT typically is prolonged, whereas the PT is normal. If the clinical symptoms suggest hemophilia B, the factor IX assay should be performed even if PTT is within the reference range, because the PTT reagent may be insensitive to mild factor IX deficiency.

Recombinant or column-purified plasma-derived factor IX concentrates are used to treat hemophilia B. Dosing is calculated the same way as for factor VIII concentrates in hemophilia A and VWD, with the exception that the calculated initial dose is doubled to compensate for factor IX distribution into the extravascular space. Repeat doses of factor IX are given every 24 hours, reflecting the half-life of the factor. The second and subsequent doses, if needed, are half the initial dose, provided that factor assays determine that the target level of factor IX was achieved.

Inhibitors to factor IX arise in only 3% of hemophilia B patients. Anti-factor IX alloantibodies react avidly with factor IX and may be detected using the Bethesda assay. Bleeding in patients with inhibitors is treated as in patients with factor VIII inhibitors using activated PCC or rFVIIa.

Hemophilia C (Rosenthal Syndrome, Factor XI Deficiency)

Factor XI deficiency is an autosomal dominant hemophilia with mild to moderate bleeding symptoms. More than half of the cases have been described in Ashkenazi Jews, but individuals of any ethnic group may be affected. The frequency and severity of bleeding episodes do not correlate with factor XI levels, and laboratory monitoring of treatment serves little purpose after the diagnosis is established. The physician treats hemophilia C with frequent plasma infusions during times of hemostatic challenge.¹¹¹ In the laboratory, the PTT is prolonged and the PT is normal.

Other Congenital Single-Factor Deficiencies

The remaining congenital single-factor deficiencies listed in Table 38-8 are rare, are caused by autosomal recessive mutations, and are often associated with consanguinity. The PT, PTT, and thrombin time may be employed to distinguish among these disorders, as shown in Table 38-7. In addition, immunoassays may be performed to distinguish among the more prevalent quantitative and the less prevalent qualitative abnormalities. In qualitative disorders, often called *dysproteinemias*,

TABLE 38-8 Rare Congenital Single-Factor Deficiencies

Deficiency	Factor Levels	Symptoms	Therapy
Afibrinogenemia	No measurable fibrinogen	Severe anatomic bleeding	
Hypofibrinogenemia	Fibrinogen activity assay <100 mg/dL	Moderate systemic bleeding	CRYO or fibrinogen concentrate to raise to >100 mg/dL
Dysfibrinogenemia	Fibrinogen activity assay <100 mg/dL	Mild systemic bleeding	
Prothrombin deficiency	Factor II <30%	Mild systemic bleeding	PCC or plasma to raise to 75%
Factor V deficiency	Factor V <30%	Mild systemic bleeding	Plasma to raise to 75%
Factor VII deficiency	Factor VII <30%	Moderate to severe anatomic bleeding	NovoSeven, PCC, four-factor PCC, plasma
Factor X deficiency	Factor X <30%	Severe anatomic bleeding	PCC or plasma to raise to 75%
Factor XI deficiency	Factor XI <50%	Anatomic bleeding	Plasma to raise to 75%
Factor XIII deficiency	Factor XIII <1%	Moderate to severe systemic bleeding, poor wound healing	Plasma or CRYO every 3 weeks

CRYO, Cryoprecipitate; PCC, prothrombin complex concentrate.

the ratio of factor activity to antigen is less than 0.7. The bleeding symptoms in the dysproteinemias may be more severe than in quantitative deficiencies, but the risk of inhibitor formation is theoretically lower. The clot-based measurement of factors II (prothrombin) and X may be supplemented or replaced by more reproducible chromogenic substrate assays. Fibrinogen is usually measured using the Clauss clot-based assay, a modification of the thrombin time, but it also may be measured by turbidimetry or immunoassay.

Because platelets transport about 20% of circulating factor V, the platelet function in *factor V deficiency* may be diminished, which is reflected in a prolonged bleeding time but normal platelet aggregation.¹¹² The PT and PTT are prolonged. Because of the concentration of factor V in platelet α -granules, normal platelet concentrate is an effective form of therapy for factor V deficiency. A combined factor V and VIII deficiency may be caused by a genetic defect traced to chromosome 18 that affects transport of both factors by a common protein in the Golgi apparatus.¹¹³

Factor VII deficiency causes moderate to severe anatomic hemorrhage. The bleeding does not necessarily reflect the factor VII activity level. The half-life of factor VII is approximately 6 hours, which affects the frequency of therapy. NovoSeven at 30 $\mu\text{g}/\text{mL}$ and nonactivated four-factor PCC preparations are effective and may provide a target factor VII level of 10% to 30%. Many factor VII deficiencies are dysproteinemias.¹¹⁴ The PT, but not the PTT, is prolonged in factor VII deficiency.

Factor X deficiency causes moderate to severe anatomic hemorrhage that may be treated with plasma or nonactivated PCC to produce therapeutic levels of 10% to 40%.¹¹⁵ The half-life of factor X is 24 to 40 hours. Acquired factor X deficiency has been described in amyloidosis, in paraproteinemia, and in association with antifungal drug therapy. The hemorrhagic symptoms may be life-threatening. The PT and PTT are both prolonged in factor X deficiency. In the *Russell viper venom time* test, which activates the coagulation mechanism at the level of

TABLE 38-9 Factor XIII Deficiency

Type of Deficiency	Incidence	Factor XIII		
		Activity	β -Protein	α -Protein
Type I	Rare	Absent	Absent	Absent
Type II	Frequent	Absent	Normal	Low
Type III	Rare	Low	Absent	Low

factor X, clotting time is prolonged in deficiencies of factors X and V, prothrombin, and fibrinogen. The venom used is harvested from the Russell viper, the most dangerous snake in Asia. This test may be useful in distinguishing a factor VII deficiency, which does not affect the results, from deficiencies in the common pathway, although specific factor assays are the standard approach.

Plasma factor XIII is a tetramer of paired α and β monomers. The intracellular form is a homodimer (two α chains) and is stored in platelets, monocytes, placenta, prostate, and uterus. The α chain contains the active enzyme site, and the β chain is a binding and stabilizing portion. Factor XIII deficiency occurs in three forms related to the affected chain, as shown in Table 38-9. Patients with factor XIII deficiency have a normal PT, PTT, and thrombin time despite anatomic bleeds and poor wound healing. They form weak (non-cross-linked) clots that dissolve within 2 hours when suspended in a 5-M urea solution, a traditional factor XIII screening assay.¹¹⁶ To confirm factor XIII deficiency, factor activity may be measured accurately using a chromogenic substrate assay such as the Behring Behrichrom FXIII assay (Behring Diagnostics, King of Prussia, PA).

Finally, autosomally inherited deficiencies of the fibrinolytic regulatory proteins α_2 -antiplasmin and plasminogen activator inhibitor-1 (PAI-1) have been reported to cause moderate to severe bleeding. Both are rare and may be diagnosed using chromogenic substrate assays.

SUMMARY

- Hemorrhage is classified as localized versus generalized, acquired versus congenital, and anatomic soft tissue hemorrhage versus systemic mucocutaneous hemorrhage.
- Acquired hemorrhagic disorders that are diagnosed in the hemostasis laboratory include thrombocytopenia of various etiologies (Chapter 40), the acute coagulopathy of trauma-shock, liver and renal disease, vitamin K deficiency, acquired hemophilia or VWD, and DIC.
- VWD is the most common congenital bleeding disorder, and the diagnosis and classification of the type and subtypes require a series of clinical laboratory assays.
- The hemophilias are congenital single-factor deficiencies that cause moderate to severe anatomic hemorrhage. The clinical laboratory plays a key role in diagnosis, classification, and treatment monitoring in hemophilia.

Now that you have completed this chapter, go back and read again the case study at the beginning and respond to the questions presented.

REVIEW QUESTIONS

Answers can be found in the Appendix.

- What is the most common acquired bleeding disorder?
 - Vitamin K deficiency
 - Liver disease
 - ACOTS
 - VWD
- Which is a typical form of anatomic bleeding?
 - Epistaxis
 - Menorrhagia
 - Hematemesis
 - Soft tissue bleed
- What factor deficiency has the speediest effect on the prothrombin time?
 - Prothrombin deficiency
 - Factor VII deficiency
 - Factor VIII deficiency
 - Factor IX deficiency
- Which of the following conditions causes a prolonged thrombin time?
 - Antithrombin deficiency
 - Prothrombin deficiency
 - Hypofibrinogenemia
 - Warfarin therapy
- In what subtype of VWD is the RIPA test result positive when ristocetin is used at a concentration of less than 0.5 mg/mL?
 - Subtype 2A
 - Subtype 2B
 - Subtype 2N
 - Type 3
- What is the typical treatment for vitamin K deficiency when the patient is bleeding?
 - Vitamin K and PCC
 - Vitamin K and plasma
 - Vitamin K and platelet concentrate
 - Vitamin K and factor VIII concentrate
- If a patient has anatomic soft tissue bleeding and poor wound healing, but the PT, PTT, thrombin time, platelet count, and platelet functional assay results are normal, what factor deficiency is indicated?
 - Fibrinogen
 - Prothrombin
 - Factor XII
 - Factor XIII
- What therapy may be used for a hemophilic boy who is bleeding and who has a high titer of factor VIII inhibitor?
 - rFVIIa
 - Plasma
 - Cryoprecipitate
 - Factor VIII concentrate
- What is the most prevalent form of VWD?
 - Type 1
 - Type 2A
 - Type 2B
 - Type 3
- Which of the following assays is used to distinguish vitamin K deficiency from liver disease?
 - PT
 - Protein C assay
 - Factor V assay
 - Factor VII assay
- Mucocutaneous hemorrhage is typical of:
 - Acquired hemorrhagic disorders
 - Localized hemorrhagic disorders
 - Defects in primary hemostasis
 - Defects in fibrinolysis

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Thrombotic Disorders and Laboratory Assessment

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OUTLINE

Developments in Thrombosis Risk Testing Etiology and Prevalence of Thrombosis

*Thrombosis Etiology
Prevalence of Thrombosis*

Thrombosis Risk Factors

Acquired Thrombosis Risk Factors

Thrombosis Risk Factors Associated with Systemic Diseases

Congenital Thrombosis Risk Factors

Double Hit

Laboratory Evaluation of Thrombophilia

*Antiphospholipid Antibodies
Activated Protein C Resistance and Factor V Leiden Mutation*

Prothrombin G20210A

Antithrombin

Protein C Control Pathway

Arterial Thrombosis Predictors

C-Reactive Protein

Plasma Homocysteine

Fibrinogen Activity

Lipoprotein (a)

Disseminated Intravascular Coagulation

Causes

Pathophysiology

Symptoms

Laboratory Diagnosis

Specialized Laboratory Tests

That May Aid in Diagnosis

Treatment

Localized Thrombosis Monitors

Heparin-Induced Thrombocytopenia

Cause and Clinical Significance

Platelet Count

Laboratory Tests for HIT

Treatment

Conclusion

OBJECTIVES

After completion of this chapter, the reader will be able to:

1. Describe the prevalence of thrombotic disease in developed countries.
2. Define thrombophilia.
3. Distinguish between venous and arterial thrombosis.
4. Differentiate among acquired thrombosis risk factors related to lifestyle and disease, and congenital risk factors, listing which factors can be assessed in the hemostasis laboratory.
5. Discuss the frequency with which the heritable risk factors occur in various ethnic groups.
6. Offer a sequence of lupus anticoagulant antibody tests that provides the greatest diagnostic validity and interpret the results of the tests.
7. Describe the relevance of antithrombin assays, proteins C and S assays, activated protein C resistance, the factor V Leiden assay, and the prothrombin G20210A assay to assess venous thrombotic risk.
8. Describe the relevance of the tests for high-sensitivity C-reactive protein, homocysteine, fibrinogen, and lipoprotein (a) to assess arterial thrombotic risk.
9. Describe the causes and pathophysiology of disseminated intravascular coagulation (DIC).
10. Describe the assays comprising a primary test profile for diagnosis and management of DIC in an acute care facility.
11. Discuss the value of quantitative D-dimer assays.
12. Describe the cause and clinical significance of heparin-induced thrombocytopenia.
13. Describe the clinical diagnosis, laboratory diagnosis, and management of heparin-induced thrombocytopenia.

CASE STUDY

After studying the material in this chapter, the reader should be able to respond to the following case study:

A 42-year-old woman with no significant medical history developed sudden onset of shortness of breath and chest pain. She was taken to an emergency department, where a pulmonary embolism was diagnosed. After admission, she was treated with intravenous heparin and given a hypercoagulability workup.

1. For what conditions can the woman be tested while she is in the hospital?
2. List possible acquired risk factors for thrombosis that need to be excluded in this patient.
3. What would be the implications of diagnosing a congenital risk factor for thrombosis in this patient?

*The author acknowledges the substantial contributions of Marisa B. Marques, MD, to Chapter 42 of the third edition of this textbook, many of which continue in this edition.

DEVELOPMENTS IN THROMBOSIS RISK TESTING

Before 1992, medical laboratory professionals performed assays to detect only three inherited venous thrombosis risk factors: deficiencies of the coagulation control factors *antithrombin*, *protein C*, and *protein S*.¹ Taken together, these three deficiencies accounted for no more than 7% of cases of recurrent venous thromboembolic disease and bore no relationship to arterial thrombosis. Since the report by Dahlback and colleagues² of *activated protein C (APC) resistance* in 1993 and the characterization by Bertina and colleagues³ of the *factor V Leiden (FVL)* mutation as its cause in 1994, efforts devoted to thrombosis prediction and evaluation have redefined the hemostasis laboratory and increased its workload exponentially. The list of current assays includes *antithrombin*, *protein C*, *protein S*, *APC resistance*, *FVL mutation*, *prothrombin G20210A mutation*, *lupus anticoagulant (LA)*, and several additional markers of both venous and arterial thrombotic disease described in this chapter. Technical improvements have enhanced the diagnostic efficacy of integrated LA detection kits, the quantitative D-dimer assay, and tests for localized coagulation activation markers such as *prothrombin fragment 1 + 2 (PF 1 + 2, PF 1.2)* and *thrombin-antithrombin (TAT) complex*.⁴

ETIOLOGY AND PREVALENCE OF THROMBOSIS

Thrombosis Etiology

Thrombosis is a multifaceted disorder resulting from abnormalities in blood flow, such as stasis, and abnormalities in the coagulation system, platelet function, leukocyte activation molecules, and the blood vessel wall. Thrombosis is the inappropriate formation of platelet or fibrin clots that obstruct blood vessels. These obstructions cause *ischemia* (loss of blood supply) and *necrosis* (tissue death).⁵

Thrombophilia (once called *hypercoagulability*) is defined as the predisposition to thrombosis secondary to a congenital or acquired disorder. The theoretical causes of thrombophilia are the following:

- Physical, chemical, or biologic events such as chronic or acute inflammation that release prothrombotic mediators from damaged blood vessels or suppress blood vessel production of normal antithrombotic substances
- Inappropriate and uncontrolled platelet activation
- Uncontrolled blood coagulation system activation
- Uncontrolled fibrinolysis suppression

Prevalence of Thrombosis

From 2000 to 2010, the U.S. death rate attributable to venous and arterial thrombotic disease declined 31%, and the number of thrombosis-related deaths declined by 17% per year. Yet, in 2010 thrombosis accounted for one of every three deaths in the United States. Of these, 25% of initial thrombotic events were fatal, and many fatal thromboses went undiagnosed before autopsy.⁶

Prevalence of Venous Thromboembolic Disease

The annual incidence of venous thromboembolic disease (or *venous thromboembolic events*, VTE) in the unselected U.S. population has remained constant for at least 25 years at 1 in 1000 and is more prevalent in African Americans and in women of childbearing age.^{7,8} The most prevalent VTE is *deep vein thrombosis*, caused by clots that form in the iliac, popliteal, and femoral veins of the calves and upper legs.⁹ Large occlusive thrombi also may form, although less often, in the veins of the upper extremities, liver, spleen, intestines, brain, and kidneys. Thrombosis symptoms include localized pain, the sensation of heat, redness, and swelling. In deep vein thrombosis, the entire leg swells.

Fragments of thrombi, called *emboli*, may separate from the proximal end of a venous thrombus, move swiftly through the right chambers of the heart, and lodge in the arterial pulmonary vasculature, causing ischemia and necrosis of lung tissue.¹⁰ Nearly 95% of these pulmonary emboli arise from thrombi in the deep leg and calf veins. Of the 250,000 U.S. residents per year who suffer pulmonary emboli, 10% to 15% die within 3 months. Many pulmonary emboli go undiagnosed because of the ambiguity of the symptoms, which may resemble those of heart disease or pneumonia. Predilection for deep venous thrombosis versus pulmonary embolism shows a familial distribution. Coagulation system imbalances, such as inappropriate activation, gain of coagulation factor function, inadequate control of thrombin generation, or suppressed fibrinolysis, are the mechanisms that cause VTE; components of cancer, or chronic heart, lung, or renal disease are often implicated in VTE.¹¹

Prevalence of Arterial Thrombosis

Cardiovascular disease caused 380,000 premature U.S. deaths in 2010, and 790,000 strokes accounted for 1 in 19 premature deaths (deaths before 78 years of age). Approximately 80% of acute myocardial infarctions and 85% of strokes are caused by thrombi that block coronary arteries or carotid end arteries of the vertebrobasilar system, respectively.¹² Transient ischemic attacks and peripheral arterial occlusions are more frequent than strokes and coronary artery disease and, although not fatal, cause substantial morbidity.

One important mechanism for arterial thrombosis is the well-described vessel wall *unstable atherosclerotic plaque*. Activated platelets, monocytes, and macrophages embed the fatty plaque within the endothelial lining, suppressing the normal release of antithrombotic molecules such as nitric oxide and exposing prothrombotic substances such as *tissue factor* (Chapter 37). Small plaques rupture, occluding arteries and releasing mediators that trigger thrombotic events. The mediators activate platelets, which combine with von Willebrand factor to form arterial platelet plugs—the “white thrombi” that cause ischemia and necrosis of surrounding tissue (Chapter 13).

The hemostasis-related lesions we associate with arterial thrombosis are blood vessel wall destruction and platelet activation. Often these are inseparable. Researchers continue to examine new thrombosis markers that capture pathological events in platelets and endothelial cells before a thrombotic event occurs.

THROMBOSIS RISK FACTORS

Acquired Thrombosis Risk Factors

In life, we acquire a legion of habits and circumstances that either help maintain or damage our hemostasis systems. Their variety and interplay make it difficult to pinpoint the factors that contribute to thrombosis or to determine which have the greatest influence. These factors seem to contribute to venous

and arterial thrombosis in varying degrees. Table 39-1 lists the nondisease risk factors implicated in thrombosis.¹³

Thrombosis Risk Factors Associated with Systemic Diseases

In addition to life events, several conditions and diseases threaten us with thrombosis. Some are listed in Table 39-2, with an indication of the laboratory's diagnostic contribution.¹⁴

TABLE 39-1 Nondisease Risk Factors That Contribute to Thrombotic Disease

Risk Factor	Comment	Contribution to Thrombosis	Laboratory Diagnosis
Age	Thrombosis after age 50	Risk doubles each decade after 50	
Immobilization	Distance driving, air travel, restriction to wheelchair or bed, obesity	Slowed blood flow raises thrombosis risk	
Diet	Fatty foods; inadequate folate, vitamin B ₆ , and vitamin B ₁₂	Homocysteinemia associated with 2× to 7× increased risk for arterial or venous thrombosis	Plasma homocysteine, vitamin levels, and lipid profile
Lipid metabolism imbalance	Hyperlipidemia, hypercholesterolemia, dyslipidemia, elevated lipoprotein (a), decreased HDL-C, elevated LDL-C	Moderate arterial thrombosis association with LDL-C elevation and hypercholesterolemia, may be congenital	Lipid profile: total cholesterol, HDL-C, LDL-C, triglycerides, and lipoprotein (a)
Oral contraceptive use	30 μg, formulation with progesterone	4× to 6× increased risk	
Pregnancy		3× to 5× increased risk	
Hormone replacement therapy		2× to 4× increased risk	
Femoral or tibial fracture		80% incidence of thrombosis if not treated with antithrombotic	
Hip, knee, gynecologic, prostate surgery		50% incidence of thrombosis if not treated with antithrombotic	
Smoking		Depends on degree	HSCRP
Inflammation	Chronic	Arterial thrombosis	HSCRP
Central venous catheter	Endothelial injury and activation	33% of children with central venous lines develop venous thrombosis	

HDL-C, High-density lipoprotein cholesterol; HSCRP, high-sensitivity C-reactive protein; LDL-C, low-density lipoprotein cholesterol.

TABLE 39-2 Diseases with Thrombotic Risk Components

Disease	Examples or Effects	Contribution to Thrombosis	Laboratory Diagnosis
Antiphospholipid syndrome	Chronic antiphospholipid antibody often secondary to autoimmune disorders	When chronic, 1.6× to 3.2× increased risk of stroke, myocardial infarction, recurrent spontaneous abortion, venous thrombosis	PTT mixing studies, lupus anticoagulant profile, anticardiolipin antibody and anti-β ₂ -glycoprotein I immunoassays
Myeloproliferative neoplasms	Essential thrombocythemia, polycythemia vera, chronic myelogenous leukemia	Increased risk due to plasma viscosity, platelet activation	Platelet counts and platelet aggregometry
Hepatic disease	Diminished production of most coagulation control proteins	Increased risk due to deranged coagulation pathways, excess thrombin production	Prothrombin time, proteins C and S, and antithrombin assays, factor assays
Cancer: adenocarcinoma	Trousseau syndrome, low-grade chronic DIC	20× increased risk of thrombosis; 10% to 20% of people with idiopathic venous thrombosis have cancer	DIC profile: platelet count, D-dimer, PTT, PT, fibrinogen, blood film examination
Leukemia	Acute promyelocytic leukemia (M3), acute monocytic leukemia, (M4-M5)	Increased risk for chronic DIC	DIC profile: platelet count, D-dimer, PTT, PT, fibrinogen, blood film examination
Paroxysmal nocturnal hemoglobinuria	Platelet-related thrombosis	Increased risk for deep vein thrombosis, pulmonary embolism, DIC	Flow cytometry phenotyping for CD55 and CD59; DIC profile: platelet count, D-dimer, PTT, PT, fibrinogen, blood film examination
Chronic inflammation	Diabetes, cancer, infection, autoimmune disorder, obesity, smoking		Fibrinogen, HSCRP

DIC, Disseminated intravascular coagulation; PT, prothrombin time; PTT, partial thromboplastin time, HSCRP, high-sensitivity C-reactive protein.

Together, transient and chronic antiphospholipid (APL) antibodies such as the *lupus anticoagulant* (LA), anticardiolipin (ACL) antibodies, and *anti- β 2-glycoprotein I* (anti- β ₂-GPI) antibodies may be detected in 1% to 2% of the unselected population.¹⁵ Chronic APL antibodies confer a risk of venous or arterial thrombosis—a condition called the *antiphospholipid syndrome* (APS). Chronic APL antibodies often accompany autoimmune connective tissue disorders, such as lupus erythematosus. Some appear in patients without any apparent underlying disease.

Malignancies often are implicated in venous thrombosis. One mechanism is tumor production of tissue factor analogues that trigger chronic low-grade disseminated intravascular coagulation (DIC). In addition, venous and arterial stasis and inflammatory effects increase the risk of thrombosis. Migratory thrombophlebitis, or *Trousseau syndrome*, is a sign of occult adenocarcinoma such as cancer of the pancreas or colon.¹⁶

Myeloproliferative neoplasms such as *essential thrombocythemia* and *polycythemia vera* (Chapter 33) may trigger thrombosis, probably through platelet hyperactivity. A cardinal sign of *acute promyelocytic leukemia* (Chapter 35) is DIC secondary to the release of procoagulant granules from malignant promyelocytes. DIC can intensify during therapy at the time of vigorous cell lysis.¹⁷ *Paroxysmal nocturnal hemoglobinuria* (PNH) (Chapter 24) is caused by a stem cell mutation that modifies membrane-anchored platelet activation suppressors. Venous or arterial thromboses occur in at least 40% of PNH cases.¹⁸

Chronic inflammatory diseases cause thrombosis through a variety of mechanisms, such as elevation of fibrinogen and factor VIII, suppressed fibrinolysis, promotion of atherosclerotic plaque formation, and reduced free protein S activity secondary to raised C4b-binding protein (C4bBP) levels. Diabetes mellitus is a particularly dangerous chronic inflammatory condition,

raising the risk of cardiovascular disease sixfold.¹⁹ Conditions associated with venous stasis, such as congestive heart failure, also are risk factors for venous thrombosis. Untreated atrial fibrillation increases the risk of ischemic strokes caused by clot formation in the right atrium and embolization to the brain.²⁰ Nephrotic syndrome creates protein imbalances that lead to thrombosis through loss of plasma proteins such as antithrombin. Nephrotic syndrome may also cause hemorrhage (Chapter 38).²¹

Congenital Thrombosis Risk Factors

Clinicians suspect congenital thrombophilia when a thrombotic event occurs in young adults; occurs in unusual sites such as the mesenteric, renal, or axillary veins; is recurrent; or occurs in a patient who has a family history of thrombosis (Table 39-3).²² Because thrombosis is multifactorial, however, even patients with congenital thrombophilia are most likely to experience thrombotic events because of a combination of constitutional and acquired conditions.²³

The *antithrombin* activity assay (previously called the antithrombin III or AT III assay) has been available since 1972, and *protein C* and *protein S* activity assays became available in the mid-1980s. The 1990s brought the *activated protein C (APC) resistance* assay and its confirmatory *factor V Leiden (FVL) mutation* molecular assay; the *prothrombin G20210A mutation* molecular assay; and tests for *dysfibrinogenemia*, *plasminogen deficiency*, *plasma TPA*, and *plasma PAI-1*.

APC resistance is found in 3% to 8% of Caucasians worldwide. APC resistance extends to Arabs and Hispanics, but the mutation is nearly absent from African and East Asian populations (Table 39-4).²⁴ APC resistance may exist in the absence of the FVL mutation and is occasionally acquired in pregnancy or in association with oral contraceptive therapy.²⁵

TABLE 39-3 Predisposing Congenital Factors and Thrombosis Risk

Risk Factor	Comment	Risk of Thrombosis	Laboratory Tests
AT (previously AT III) deficiency	AT, enhanced by heparin, inhibits the serine proteases IIa (thrombin), IXa, Xa, and XIa	Heterozygous: increased 10× to 20× Homozygous: 100%, rarely reported	Clot-based and chromogenic AT activity assays, immunoassays for AT concentration (antigen)
PC deficiency	Activated PC is a serine protease that hydrolyzes factors Va and VIIIa, requires protein S as a stabilizing cofactor	Heterozygous: increased 2× to 5× Homozygous: 100%; causes neonatal purpura fulminans	Clot-based and chromogenic PC activity assays, immunoassay for PC concentration (antigen)
Free PS deficiency	PS is a stabilizing cofactor for activated protein C, 40% is free, 60% circulates bound to C4bBP	Heterozygous: increased 1.6× to 11.5× Homozygous: 100% but rarely reported; causes neonatal purpura fulminans	Clot-based free PS activity assays, free and total PS immunoassays for PS concentration (antigen)
APC resistance	Factor V Leiden (R506Q) mutation gain of function renders factor V resistant to APC	Heterozygous: increased 3× Homozygous: increased 18×	PTT-based APC resistance test and confirmatory molecular assay
Prothrombin G20210A	Mutation in prothrombin gene untranslated 3' promoter region creates moderate elevation in prothrombin activity	Heterozygous: increased 1.6× to 11.5×	Molecular assay only; phenotypic assay provides no specificity
Hyperfibrinogenemia	Associated with arterial thrombosis	Under investigation: acute phase reactant	Clauss fibrinogen clotting assay, immunoassay, nephelometric assay

APC, Activated protein C; AT, antithrombin; C4bBP, complement component C4b binding protein; PC, protein C; PS, protein S; PTT, partial thromboplastin time.

TABLE 39-4 Prevalence of Congenital Thrombosis Risk Factors in the General Population and in Individuals with Recurrent Thrombotic Disease

Factor	Unselected Population	People with at Least One Thrombotic Event
Activated protein C resistance, factor V Leiden mutation	3% to 8% of Caucasians, rare in Asians or Africans	20%–25%
Prothrombin G20210A	2% to 3% of Caucasians, rare in Asians or Africans	4%–8%
Antithrombin deficiency	1 in 2000 to 1 in 5000	1%–1.8%
Protein C deficiency	1 in 300	2.5%–5.0%
Protein S deficiency	Unknown	2.8%–5.0%
Hyperhomocysteinemia associated with methylenetetrahydrofolate reductase gene mutations	11%	13.1%–26.7%

The FVL gene mutation is the most common inherited thrombophilia, and the prothrombin G20210A gene mutation is the second most common inherited thrombophilia tendency in patients with a personal and family history of deep vein thrombosis.²⁶ Altogether, protein C, protein S, and antithrombin deficiencies are found in only 0.2% to 1.0% of the world population. The incidences of dysfibrinogenemia and the various forms of abnormal fibrinolysis (plasminogen deficiency, TPA deficiency, and PAI-1 excess) are under investigation.

Double Hit

Thrombosis often is associated with a combination of genetic defect, disease, and lifestyle influences. Just because someone possesses protein C, protein S, or antithrombin deficiency does not mean that thrombosis is inevitable. Many heterozygotes experience no thrombotic event during their lifetimes, whereas others experience clotting only when two or more risk factors

converge. A young woman who is heterozygous for the FVL mutation has a thirty-fivefold increase in thrombosis risk upon starting oral contraceptive therapy. In the Physicians' Health Study, homocysteinemia tripled the risk of idiopathic venous thrombosis, and the FVL mutation doubled it. When both were present, the risk of venous thrombosis was increased tenfold.²⁷

LABORATORY EVALUATION OF THROMBOPHILIA

When thrombophilia is suspected, it is important to assess all known risk factors because it is the combination of positive results that determines the patient's cumulative risk of thrombosis.²⁸ Table 39-5 summarizes the commonly used thrombosis risk assays and indicates those that can be relied on while the patient is on antithrombotic therapy or while the patient is recovering from an acute thrombotic event.

TABLE 39-5 Thrombophilia Laboratory Test Profile

Assay	Reference Value/Interval	Comments
APC resistance	Ratio ≥ 1.8	Clot-based screen that employs PTT with factor V-depleted plasma.
Factor V Leiden mutation	Wild-type	Molecular assay performed as follow-up to APC resistance ratio that is < 1.8 .
Prothrombin G20210A	Wild-type	Molecular assay. There is no phenotypic assay for prothrombin G20210A.
LA profile*	Negative for LA	Minimum of two clot-based assays. Primary assays are based on PTT and DRVVT, secondary assays based on KCT, or dilute PT. All four include phospholipid neutralization follow-up test.
ACL antibody	IgG: < 12 GPL IgM: < 10 MPL	Immunoassay for immunoglobulins of APL family. ACL depends on β_2 -GPI in reaction mix.
Anti- β_2 -GPI antibody	< 20 G units	Immunoassay for an immunoglobulin of APL family. β_2 -GPI is key phospholipid-binding protein in family.
AT activity*	78%–126%	Serine protease inhibitor suppresses IIa (thrombin), IXa, Xa, XIa. When consistently below reference limit, follow up with AT antigen assay.
PC activity*	70%–140%	Digests VIIIa and Va. When consistently below reference limit, follow up with PC antigen assay.
PS activity*	65%–140%	PC cofactor. When consistently below reference limit, follow up with total and free PS antigen assay, C4b-binding protein assay.
Fibrinogen	220–498 mg/dL	Clot-based assay. Elevation may be associated with arterial thrombosis.

ACL, anticardiolipin; APC, activated protein C; APL, antiphospholipid antibody; AT, antithrombin; β_2 -GPI, β_2 -glycoprotein I; DRVVT, dilute Russell viper venom time; GPL, IgG antiphospholipid antibody unit; Ig, immunoglobulin; KCT, kaolin clotting time; LA, lupus anticoagulant; MPL, IgM antiphospholipid antibody unit; PC, protein C; PS, protein S; PT, prothrombin time; PTT, partial thromboplastin time.

*Inaccurate during active thrombosis or anticoagulant therapy. Perform 14 days after anticoagulant therapy is discontinued.

The presence or absence of laboratory-detected risk factors does not affect anticoagulant treatment when thrombosis is in progress.²⁹ However, it is important to realize that current anticoagulant therapy and ongoing or recent thrombotic events interfere with the interpretation of antithrombin, protein C, protein S, factor VIII, and LA testing. These assays should be performed at least 14 days after anticoagulant therapy is discontinued.

Antiphospholipid Antibodies

APL antibodies comprise a family of immunoglobulins that bind protein-phospholipid complexes.³⁰ APL antibodies include LAs, detected by clot-based profiles, and ACL and anti- β_2 -GPI antibodies, detected by immunoassay. Chronic autoimmune APL antibodies are associated with APS, which is characterized by transient ischemic attacks, strokes, coronary and peripheral artery disease, venous thromboembolism, and recurrent pregnancy complications, including spontaneous abortions.^{31,32}

APL antibodies arise as immunoglobulin M (IgM), IgG, or IgA isotypes. Because they may bind a variety of protein-phospholipid complexes, they are called *nonspecific inhibitors*. Their name implies that they were once thought to directly bind phospholipids; however, their target antigens are actually the proteins that assemble on anionic phospholipid surfaces.³³ The plasma protein most often bound by APL antibodies is β_2 -GPI, although *annexin V* and *prothrombin* are sometimes implicated as APL targets. APL antibodies probably develop in response to newly formed protein-phospholipid complexes, and laboratory scientists continue to investigate how they cause thrombosis.^{34,35}

Clinical Consequences of Antiphospholipid Antibodies

Between 1% and 2% of unselected individuals of both sexes and all races, and 5% to 15% of individuals with recurrent venous or arterial thrombotic disease have APL antibodies.³⁶ Most APL antibodies arise in response to a bacterial, viral, fungal, or parasitic infection or to treatment with numerous drugs (Box 39-1) and disappear within 12 weeks. These are mostly transient *alloimmune* APL antibodies and have no clinical consequences.³⁷ Nevertheless, the laboratory professional must follow up any positive results on APL antibody assays to determine their persistence.

BOX 39-1 Agents Known to Induce Antiphospholipid Antibodies

Various antibiotics
Phenothiazine
Hydralazine
Quinine and quinidine
Calcium channel blockers
Procainamide
Phenytoin
Cocaine
Elevated estrogens

Autoimmune APL antibodies are part of the family of autoantibodies that arise in collagen vascular diseases; 50% of patients with systemic lupus erythematosus have autoimmune APL antibodies. Autoimmune APL antibodies are also detected in patients with rheumatoid arthritis, scleroderma, and Sjögren syndrome but may arise spontaneously, a disorder called *primary APS*. Autoimmune APL antibodies may persist, and fully 30% are associated with arterial and venous thrombosis. Chronic presence of an autoimmune APL antibody not associated with a known underlying autoimmune disorder confers a 1.8-fold to 3.2-fold increased risk of thrombosis.

Detection and Confirmation of Antiphospholipid Antibodies

Clinicians suspect APS in unexplained venous or arterial thrombosis, thrombocytopenia, or recurrent fetal loss.³⁸ Specialized clinical hemostasis laboratories offer APL detection profiles that include clot-based assays for LA and immunoassays for ACL and anti- β_2 -GPI antibodies. Occasionally, an LA is suspected because of an unexplained prolonged partial thromboplastin time (PTT) that does not correct in mixing studies (Figure 39-1; Chapter 42).^{39,40}

Lupus Anticoagulant Test Profile

Clot-based assays with reduced reagent phospholipid concentrations are sensitive to LA. There are two commonly used test systems, and both are required for an LA profile. The need for two parallel assay systems arises from the multiplicity of LA reaction characteristics: a confirmed positive result in one system is conclusive despite a negative result in the other. The two most commonly recommended test systems are the dilute Russell viper venom time (DRVVT) and the silica-based partial thromboplastin time (PTT), both formulated with low-phospholipid concentrations designed to be LA sensitive.⁴¹ Two older systems, still used in many institutions and available from specialty laboratories and coagulation reagent distributors, are the kaolin clotting time (KCT) and the dilute thromboplastin time (DTT, also named tissue thromboplastin inhibitor test, TTI).⁴² As illustrated in Figure 39-2, the KCT and PTT initiate coagulation at the level of factor XII; DRVVT at factor X; and DTT at factor VII.

The 2009 International Society on Thrombosis and Haemostasis (ISTH) update of guidelines for LA detection provides the following sequence of assays:⁴³

1. Prolonged phospholipid-dependent clot formation using an initial screen assay such as a low phospholipid PTT or DRVVT.
2. Failure to correct the prolonged clot time when mixing with normal platelet-poor control plasma and repeating the test (see mixing study below).
3. Shortening or complete correction of the prolonged screen assay result by addition of a reagent formulated with excess phospholipids.
4. Exclusion of other coagulopathies.

Performing the Clot-Based Lupus Anticoagulant Mixing Study. Laboratory practitioners perform the LA profile upon clinician request, often based on adverse thrombotic or obstetric

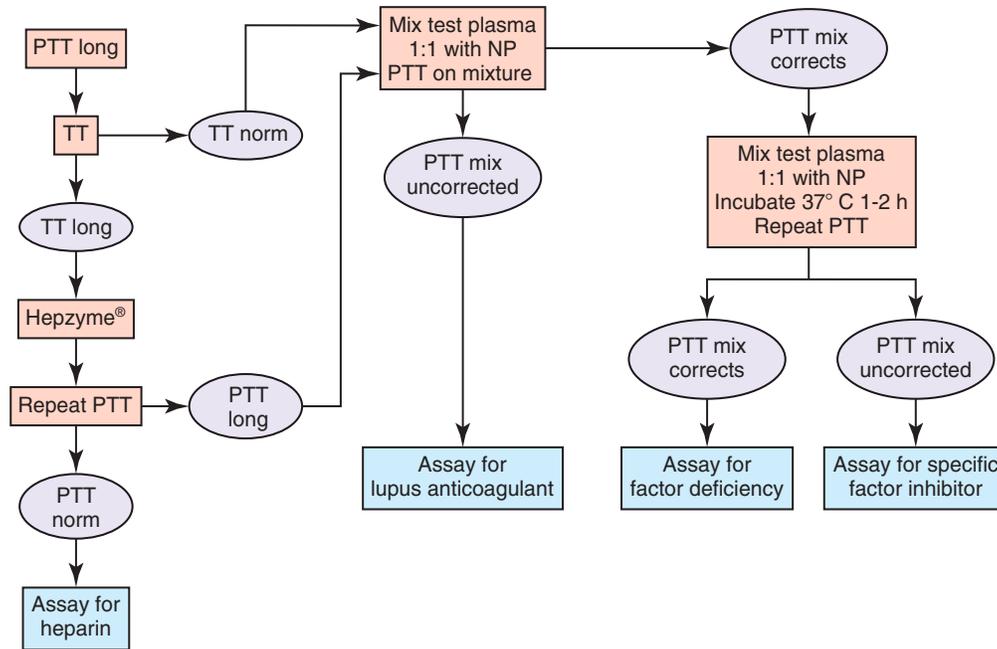


Figure 39-1 Mixing study employing a partial thromboplastin time (PTT) reagent with intermediate lupus anticoagulant sensitivity. Beginning at the top left, when the PTT result exceeds the upper limit of the PTT reference interval, perform a thrombin clotting time (TT) to detect unfractionated heparin. If the TT exceeds the TT reference interval, presume that heparin is present. Treat an aliquot of the specimen with Hepzyme and repeat the PTT. If the new PTT is normal, assay the original sample for heparin. If the PTT remains prolonged, or if the TT was normal, proceed by mixing the patient plasma with control normal plasma (NP) and perform a PTT on the mixture. If the PTT mixture corrects, prepare a new 1:1 mix and incubate at 37° C for 1 to 2 hours and repeat the PTT, comparing the result to incubated NP. If the incubated PTT shows a correction, assay for a factor deficiency. If prolonged, assay for a lupus anticoagulant (Figs. 39-2 and 39-3) (a specific factor inhibitor such as anti-factor VIII associated with bleeding will also be detected in this manner).

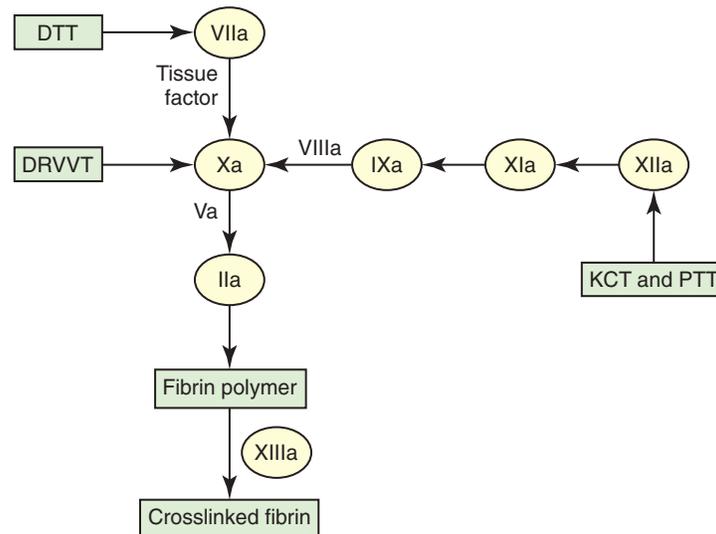


Figure 39-2 Simplified coagulation pathway illustrating the activation points of the lupus anticoagulant (LA) assays. The dilute Russell viper venom time (DRVVT) assay, regarded as the primary LA detection method, activates factor X (ten). DRVVT is typically accompanied by a low-phospholipid LA-sensitive partial thromboplastin time assay (PTT) that activates factor XII. The historic kaolin clotting time (KCT) also activates factor XII, and the dilute thromboplastin time test (DTT), still available, activates factor VII.

events, or when a prolonged PTT raises the presumption of an LA. Most laboratory protocols begin with a mixing study where the PTT is performed on patient plasma combined with control normal plasma (NP) (Figure 39-1; Chapter 42). The mixing study includes a 37° C incubation step, as many LAs and specific inhibitors require an incubation to enhance their

avidity. Practitioners typically perform the mixing study using a PTT reagent with intermediate LA sensitivity but may substitute the prothrombin time (PT) reagent, DRVVT, or an LA-sensitive low-phospholipid PTT reagent. The mixing study includes a means for detecting unfractionated heparin, most often the thrombin clotting time or the chromogenic anti-Xa

heparin assay. The practitioner may add heparinase (Hepzyme®, Siemens Healthcare USA, Inc., Malvern, PA) to the sample to neutralize heparin, although this may be unnecessary because many LA detection reagents provide heparin-neutralizing polybrene. Mixing studies that use a PT, a DRVVT, or an LA-sensitive PTT reagent seldom include the 37° C incubation step.

The NP that is mixed 1:1 with patient plasma will shorten a prolonged PTT. Each laboratory director decides what degree of PTT “shortening” constitutes “correction.” Many use the Rosner index, which defines correction as a mixture result within 10% of the NP result.⁴⁴ Others define correction as return to a value within 5 seconds of the NP result or return to a value within the PTT reference interval.

In performing mixing studies, the laboratory professional employs only platelet-poor plasma—plasma centrifuged so that it has a platelet count of less than 10,000/ μ L (Chapter 42). The use of platelet-poor plasma avoids neutralization of LA by the platelet membrane phospholipids. Platelet membrane fragments that form during freezing and thawing can likewise neutralize LA and lead to a false-negative LA result.

Performing Clot-Based Lupus Anticoagulant Tests.

Following a mixing study that suggests the presence of an LA, specific testing for the LA is performed. Most LA protocols begin with the DRVVT, considered the most specific of the LA assays (Figure 39-3). If the DRVVT screening reagent-patient plasma result exceeds the NP result by a predetermined ratio, frequently near 1.2, LA is presumed. The practitioner then confirms LA by mixing an aliquot of the patient sample with the DRVVT high-phospholipid confirmatory reagent, comparing the result in seconds to the original DRVVT screening reagent patient plasma result. The reagent phospholipid neutralizes LA and shortens the DRVVT. If the original screening reagent result exceeds the DRVVT confirm reagent result by a predetermined ratio, again near 1.2, LA is confirmed.

If the original DRVVT screen ratio was less than 1.2, the practitioner turns to the silica-based low-phospholipid LA-sensitive PTT and repeats the steps used for the DRVVT, again basing results on a predetermined ratio, often 1.2.^{45,46}

There exist numerous modifications to this algorithm. Many laboratory directors prefer to begin with the silica-based low-phospholipid PTT assay, and others include an intermediate NP mixing study step. Some incorporate the KCT or DTT. Some assay systems use an absolute difference, in seconds, instead of a ratio, often 8 seconds. The 1.2 ratio and the 8-second difference are examples; each institution establishes its own reference interval and threshold ratio or difference.

Some laboratory directors choose to normalize ratios using the mean of the reference interval (MRI) or the NP value. The formula for normalization using the MRI is:

$$\text{Normalized ratio} = \frac{\text{Patient screen result in seconds/screen MRI}}{\text{Patient confirm result in seconds/confirm MRI}} = \frac{\text{Screen ratio}}{\text{Confirm ratio}}$$

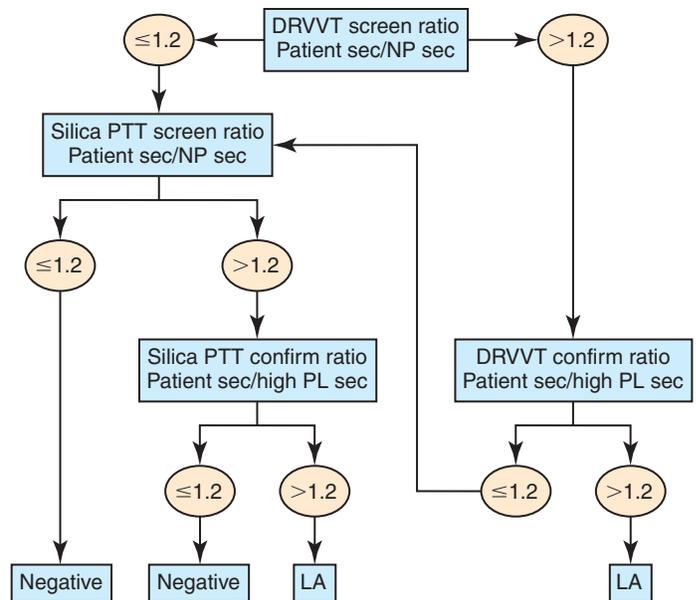


Figure 39-3 Lupus anticoagulant (LA) algorithm. When LA is suspected, perform a dilute Russell viper venom (DRVVT) screen, comparing the patient DRVVT screen result to the control normal plasma (NP) DRVVT screen result. If the ratio of patient to NP DRVVT in seconds is greater than 1.2, mix patient plasma 1:1 with high-phospholipid DRVVT confirm reagent and perform a new DRVVT. If the patient DRVVT screen result exceeds the DRVVT confirm/patient plasma result by greater than 1.2, LA is confirmed. If the ratio is 1.2 or less or if the original DRVVT screen ratio was 1.2 or less, proceed to a silica-based partial thromboplastin (PTT) screen and confirm. Compare the patient silica-based PTT screen to the NP screen result. If the ratio is 1.2 or less, no LA is present. If the ratio is greater than 1.2, mix the patient plasma with high-phospholipid silica-based PTT confirm reagent and perform a new PTT. If the patient PTT screen exceeds the patient PTT confirm by more than 1.2, LA is confirmed. If the ratio is 1.2 or less, LA is not present.

Anticardiolipin Antibody Immunoassay

LA and ACL antibodies coexist in 60% of cases, and both may be found in APS. The ACL test is an immunoassay that may be normalized among laboratories and is not affected by heparin therapy, oral anticoagulant therapy, current thrombosis, or factor deficiencies.

The manufacturer coats microplate wells with bovine heart cardiolipin and blocks (fills open receptor sites) with a bovine serum solution containing β_2 -GPI. The laboratory practitioner pipettes test sera or plasmas to the wells alongside calibrators and controls (Figure 39-4). ACL binds the solid-phase cardiolipin- β_2 -GPI target complex and cannot be washed from the wells. The practitioner adds enzyme-labeled anti-human IgG, IgM, or IgA conjugates subsequent to washing, followed by a color-producing substrate. A color change indicates the presence of ACL and color intensities of the patient, and control sample wells are compared with the calibrator curve wells. Results are expressed using GPL, MPL, or APL units, where 1 unit is equivalent to 1 μ g/mL of an affinity-purified standard IgG, IgM, or IgA specimen.⁴⁷ Reference limits are established in each laboratory.⁴⁸

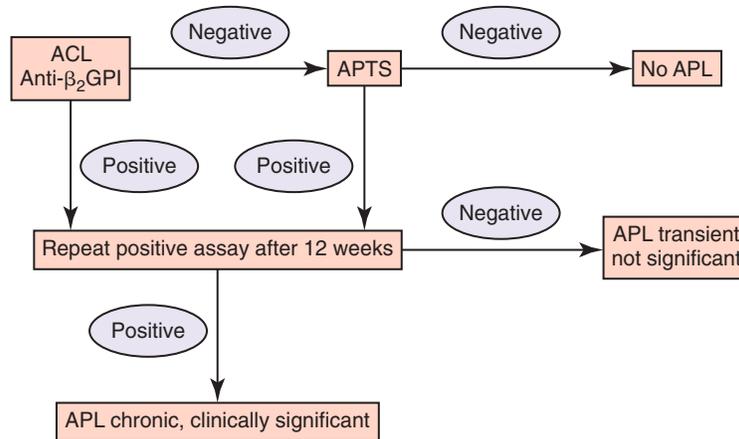


Figure 39-4 Antiphospholipid antibody (APL) immunoassay algorithm. If an APL is suspected, perform an anticardiolipin (ACL) or anti- β_2 -glycoprotein I (anti- β_2 -GPI) immunoassay. If either is positive, confirm chronicity using a new specimen collected at least 12 weeks later. If negative, perform immunoassay to detect an antiphosphatidyl serine (APTS) antibody. If positive, repeat after 12 weeks.

Anti- β_2 -Glycoprotein I Immunoassay

The practitioner performs IgM and IgG anti- β_2 -GPI immunoassays as a part of the profile that includes ACL assays. An anti- β_2 -GPI result of greater than 20 IgG or IgM anti- β_2 -GPI units correlates with thrombosis more closely than the presence of ACL antibodies. Any ACL or anti- β_2 -GPI assay yielding positive results is repeated on a new specimen collected after 12 weeks to distinguish a transient alloantibody from a chronic autoantibody.

Antiphosphatidylserine Immunoassay

For cases in which an APL antibody is suspected but the routine LA, ACL, and β_2 -GPI assay results are negative, the clinician may wish to order the antiphosphatidylserine immunoassay to detect APL antibodies specific for phosphatidylserine.⁴⁹ A result greater than or equal to 16 IgG or 22 IgM antiphosphatidylserine units is considered positive. The antiphosphatidylserine assay is available from specialty reference laboratories.

Activated Protein C Resistance and Factor V Leiden Mutation

Clinical Importance of Activated Protein C Resistance

The *activated protein C* (APC)–protein S complex normally hydrolyzes activated factors V and VIII (factors Va and VIIIa). A mutation in the factor V gene substitutes glutamine for arginine at position 506 of the factor V molecule (FV R506Q). The arginine molecule is a normal cleavage site for APC, so the substitution slows or resists APC hydrolysis (Figure 39-5). The resistant factor Va remains active and raises the production of thrombin, leading to thrombosis. The factor V R506Q mutation is named for the city in The Netherlands in which it was first described: Leiden [*factor V Leiden* (FVL) mutation, also referred to as *APC resistance*]. Between 3% and 8% of Northern European Caucasians possess the FVL mutation (Table 39-4).⁵⁰ Owing to its prevalence and the associated threefold higher

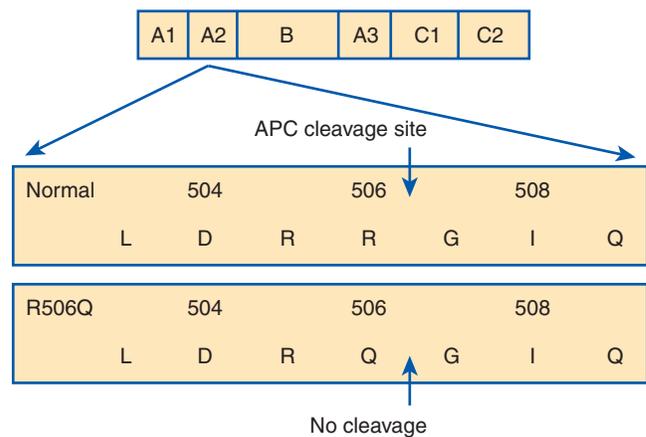


Figure 39-5 Factor V Leiden mutation. A point mutation within the factor V gene results in the substitution of amino acid glutamine for arginine at position 506 (R506Q) of the factor V molecule. The normal arginine at position 506 is a cleavage site for activated protein C (APC), so glutamine substitution slows or prevents cleavage of the factor V molecule. *D*, Aspartic acid; *G*, glycine; *I*, isoleucine; *L*, leucine; *R*, arginine, *Q*, glutamine.

thrombosis risk (eighteenfold higher for homozygotes), most acute care hemostasis laboratory directors provide APC resistance detection to screen for FVL.^{51,52}

Activated Protein C Resistance Clot-Based Assay

In the APC resistance clot-based assay, patient plasma is mixed 1:4 with factor V–depleted plasma.⁵³ PTT reagent is added to two aliquots of the mixture and incubated for 3 minutes (Figure 39-6). A solution of calcium chloride is pipetted into one mixture, and the clot formation is timed. A solution of *calcium chloride with APC* is added to the second mixture, and clotting is timed. The time to clot formation of the second aliquot is at least 1.8 times the time to clot formation of the first (prolonged time to clot due to the increased amount of APC), so the normal ratio of PTT results between the two assays is 1.8 or greater. In APC resistance, the ratio is less than 1.8.⁵⁴

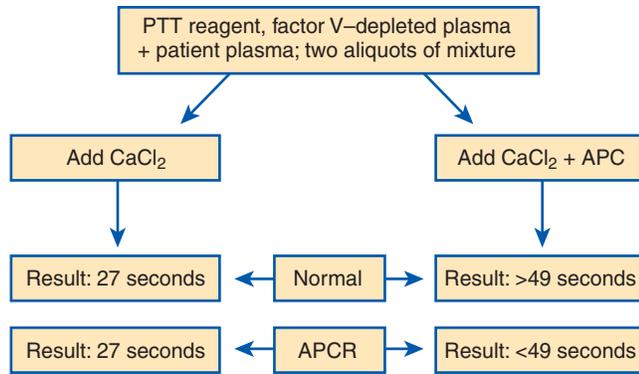


Figure 39-6 Activated protein C resistance ratio (APCR) measurement. Patient plasma is mixed 1:4 with factor V–depleted plasma and partial thromboplastin time (PTT) reagent. Two aliquots of this mixture are then tested: one aliquot is mixed with calcium chloride (CaCl₂) alone, and the other aliquot is mixed with CaCl₂ plus activated protein C (APC). The reaction in the mixture with the APC should be prolonged to a clotting time at least 1.8 times longer than the mixture without APC. A ratio of 1.8 or less implies APC resistance.

Performance Characteristics of the Activated Protein C Resistance Test. Factor V–depleted plasma compensates for potential factor deficiencies and for oral anticoagulant therapy by providing normal coagulation factors. The laboratory professional uses only platelet-poor plasma in the APC resistance test to prevent loss of sensitivity caused by the abundant release of platelet factor 4. APC resistance reagent test kits contain polybrene or heparinase to neutralize UFH. LA, however, affects the test system adversely.⁵⁵ If LA is present, the molecular test for FVL is indicated.⁵⁶

Factor V Leiden Mutation Assay

Most laboratories confirm the APC resistance diagnosis using the molecular FVL mutation test. The determination of zygosity is important to predict the risk for thrombosis and establish a treatment regimen.

Prothrombin G20210A

A guanine-to-adenine mutation at base 20210 of the 3' untranslated region of the *prothrombin* (factor II) gene has been associated with mildly elevated plasma prothrombin levels, averaging 130%.⁵² The increased risk of thrombosis in those with the mutation seems to be related to the elevated prothrombin activity.⁵³ The prevalence of this mutation among individuals with familial thrombosis is 5% to 18%, whereas prevalence worldwide is 0.3% to 2.4%, depending on race.⁵⁷ The risk of venous thrombosis in heterozygotes is only two to three times the baseline risk. Although the mutation may cause a slight prothrombin elevation, a phenotypic prothrombin activity assay is of little diagnostic value because there is considerable overlap between normal prothrombin levels and prothrombin levels in people with the mutation.^{58,59}

Antithrombin

Antithrombin (previously named antithrombin III or ATIII) is a serine protease inhibitor (SERPIN) that neutralizes factors IIa

(thrombin), IXa, Xa, XIa, and XIIa, all the serine proteases except factor VIIa. Antithrombin activity is enhanced by unfractionated heparin (UFH), low-molecular-weight heparin (LMWH), and synthetic pentasaccharide (fondaparinux) (Figures 39-7 and 39-8). Antithrombin was the first of the plasma coagulation control proteins to be identified and the first to be assayed

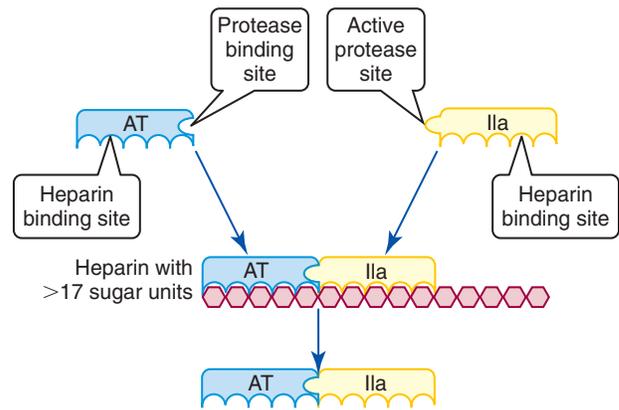


Figure 39-7 The reaction between antithrombin (AT) and activated coagulation factor II (IIa; thrombin) is supported by heparin. Standard unfractionated heparin, with a molecular weight of 5000 to 40,000 Daltons, provides polysaccharide chains of at least 17 sugar subunits. Heparin molecules of this length support the reaction between AT and IIa, as AT and IIa assemble on the heparin molecule. The AT molecule attaches to a specific pentasaccharide sequence. The IIa possesses a heparin-binding site that enables it to assemble on the heparin surface adjacent to the AT, a property called *approximation*. The AT becomes sterically modified (allosteric), supporting a covalent reaction between the AT protease binding site and the IIa active protease site. Thrombin (IIa) and antithrombin, covalently bound, release from heparin and form measurable plasma thrombin-antithrombin (TAT) complexes, useful as a marker of coagulation activation.

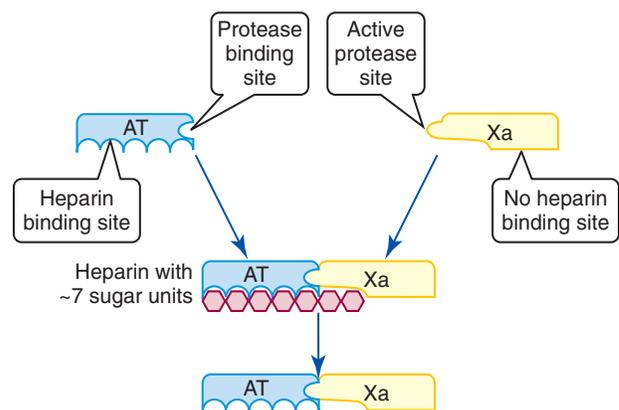


Figure 39-8 The reaction between antithrombin (AT) and activated coagulation factor X (Xa) is supported by low-molecular-weight heparin. Low-molecular-weight heparin, with a molecular weight of less than 8000 Daltons, provides polysaccharide chains of six or seven sugar subunits. Heparin molecules of this length support the reaction between AT and Xa. The AT molecule attaches to a specific pentasaccharide sequence. The Xa does not possess a heparin-binding site and does not need to assemble on the heparin surface adjacent to the AT. The AT becomes sterically modified (allosteric), supporting a covalent reaction between the AT protease binding site and the Xa active protease site.

routinely in the clinical hemostasis laboratory.⁵⁵ Other members of the serpin family are heparin cofactor II, α_2 -macroglobulin, α_2 -antiplasmin, and α_1 -antitrypsin. Typically, hemostasis specialty laboratories or reference laboratories are the only places that assay serpins other than the more commonly ordered antithrombin activity, which has found its way into laboratories at most acute care facilities.

Antithrombin Deficiency

Acquired antithrombin deficiency occurs in liver disease, in nephrotic syndrome, with prolonged heparin therapy, with asparaginase therapy, with the use of oral contraceptives, and in DIC, where antithrombin is rapidly consumed. Congenital deficiency is present in 1 in 2000 to 1 in 5000 of the general population and accounts for 1.0% to 1.8% of recurrent venous thromboembolic disease cases.⁶⁰ About 90% of cases of antithrombin deficiency are quantitative (reduced production), or type I; the remainder are caused by mutations creating structural abnormalities in the antithrombin *protease binding* site or the *heparin binding* site. Type II mutations do not reduce antithrombin production, but the molecules are nonfunctional.⁶¹

Antithrombin Reference Intervals

Adult plasma antithrombin activity ranges from 78% to 126%, whether measured by clot-based or chromogenic assay. Antithrombin antigen levels range from 22 to 39 mg/dL (68% to 128%) by latex microparticle immunoassay, and the plasma biologic half-life is 72 hours. Adult levels are reached by 3 months of age, and levels remain steady throughout adult life, except during periods of physiologic challenge, such as pregnancy. Antithrombin activity decreases with age.⁶²

Antithrombin Activity Assay

Laboratory practitioners screen for antithrombin deficiency using a clot-based or chromogenic functional assay. The clot-based assay has been available since 1972, but most laboratory directors choose the chromogenic assay because of its stability and reproducibility. The operator mixes test plasma with a solution of heparin and factor Xa (Figure 39-9) and incubates the mixture at 37° C for several minutes. During incubation, the heparin-activated plasma antithrombin irreversibly binds a proportion of the reagent factor Xa. Residual factor Xa hydrolyzes a chromogenic substrate, added as a second reagent. The degree of hydrolysis, measurable by colored end product intensity, is inversely proportional to the activity of antithrombin in the test plasma.⁶³ The chromogenic substrate test for plasma antithrombin activity detects quantitative and qualitative antithrombin deficiencies and detects mutations affecting the proteolytic site but not the heparin binding site. Clot-based antithrombin assays are affected by, and therefore detect, mutations in both the proteolytic and heparin binding sites.

Antithrombin Antigen Assay

Antithrombin concentration is measured in a turbidometric microparticle immunoassay using a suspension of latex microbeads coated with antibody to antithrombin. In the absence of antithrombin, the wavelength of incident monochromatic

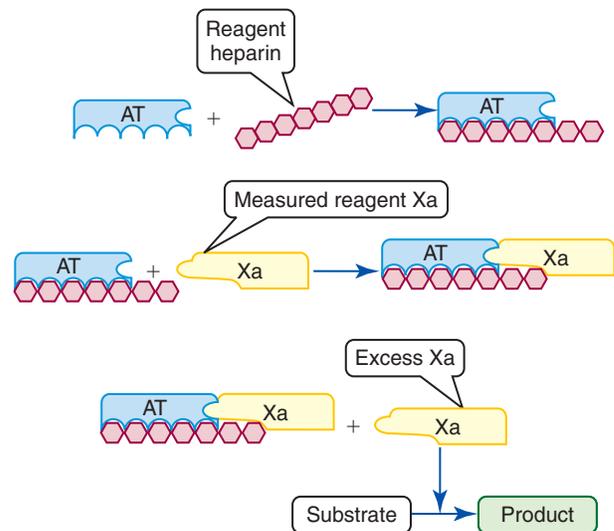


Figure 39-9 Chromogenic antithrombin (AT) functional assay. Patient plasma is pipetted into a reagent consisting of heparin, a measured concentration of activated coagulation factor X (Xa), and a chromogenic substrate. AT is activated by heparin and binds Xa. Excess Xa hydrolyzes the substrate and produces a yellow product, para-nitroaniline (pNA). The intensity of pNA color is inversely proportional to AT activity.

light exceeds the latex microparticle diameter, so the light passes through unabsorbed.⁶⁴ In the presence of antithrombin, the particles form larger aggregates. The antithrombin concentration is directly proportional to the rate of light absorption change. Antithrombin antigen levels are diminished in quantitative (type I) but not qualitative (type II) antithrombin deficiency. Oral anticoagulant Coumadin therapy may raise the antithrombin level and mask a mild deficiency. Antithrombin activity remains decreased for 10 to 14 days after surgery or a thrombotic event, so the assay should not be used to establish a congenital deficiency during this period.⁶⁵

Heparin Resistance and the Antithrombin Assay

Antithrombin may become decreased during prolonged or intense heparin therapy and may be largely consumed if the patient has a congenital antithrombin deficiency. In this instance, heparin may be administered in therapeutic or higher dosages, but it neither exerts an anticoagulant effect nor is detected by the PTT. This is known as *heparin resistance*. In such cases, an antithrombin assay is necessary to confirm antithrombin deficiency. Antithrombin deficiency may be treated with antithrombin concentrate (Thrombate III; Grifols, Inc., Los Angeles, CA).⁶⁶

Protein C Control Pathway

Thrombin is an important coagulation factor because it cleaves fibrinogen, activates platelets, and activates factors V, VIII, XI, and XIII. In the intact vessel where clotting would be pathological, thrombin binds endothelial cell membrane *thrombomodulin* and becomes an anticoagulant.⁶⁷ How does this paradox happen? The thrombin–thrombomodulin complex activates plasma protein C, and the APC binds free plasma protein S (Figure 39-10).⁶⁸ The stabilized APC–protein S complex, simultaneously bound to

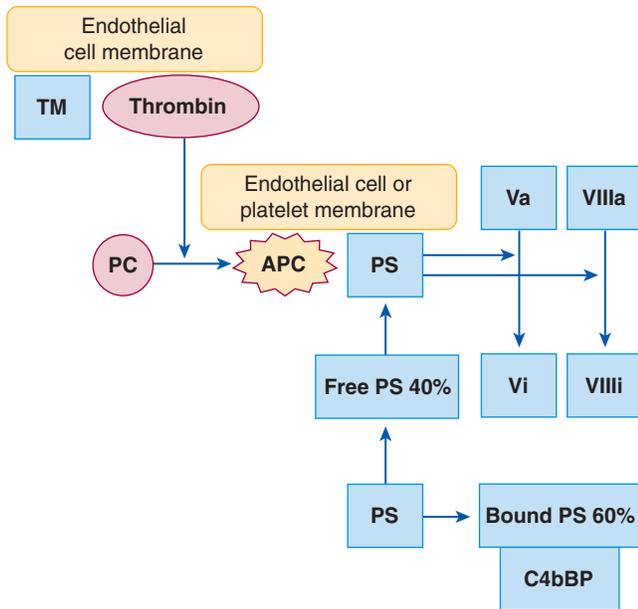


Figure 39-10 Protein C pathway. Thrombin (activated coagulation factor II or IIa) binds constitutive thrombomodulin (TM) on the endothelial cell membrane. The TM-thrombin complex activates protein C (PC). Activated protein C (APC) binds protein S (PS) on endothelial cell or platelet membrane phospholipids. The bound active complex hydrolyzes (inactivates) activated coagulation factors V and VIII (Va and VIIIa). PS can be in either a free (able to interact as described above) or bound state. *C4bBP*, C4b-binding protein; *Vi*, inactivated coagulation factor V; *VIIIi*, inactivated coagulation factor VIII.

the endothelial protein C receptor, hydrolyzes factors Va and VIIIa to slow coagulation. Recurrent venous thrombosis is the potential consequence of protein C or protein S deficiency.⁶⁹

Protein S, the cofactor that binds and stabilizes APC, circulates in the plasma either free or covalently bound to the complement binding protein *C4bBP*. Bound protein S cannot participate in the protein C anticoagulant pathway; only free plasma protein S is available to serve as the APC cofactor. Protein S–*C4bBP* binding is of particular interest in inflammatory conditions, where acute phase reactant *C4bBP* level rises, binding additional protein S. Free protein S levels are proportionally decreased.

Protein C and Protein S Reference Ranges

Heterozygous protein C or protein S deficiency leads to a 1.6-fold to 11.5-fold increased risk of recurrent deep vein thrombosis and pulmonary embolism. Protein S deficiency also has been implicated in transient ischemic attacks and strokes, particularly in the young. The reference interval for both protein C and protein S activity and antigen levels is 65% to 140%, and levels ordinarily remain between 30% and 65% for heterozygotes.

Control proteins C and S (and Z) and factors II (prothrombin), VII, IX, and X are vitamin K-dependent. Because the half-life of protein C is 6 hours, its level decreases as rapidly as that of factor VII at the onset of Coumadin (warfarin, vitamin K antagonist) therapy. In heterozygous protein C deficiency, protein C activity may drop to less than 65% (a thrombotic level) more rapidly than the coagulation factor activities reach low

therapeutic levels (less than 30%). Consequently, in early Coumadin therapy a patient may experience *Coumadin-induced skin necrosis*, a paradoxical situation in which the anticoagulant therapy brings on thrombosis of the dermal vessels. This complication is suspected when the patient develops painful necrotic lesions that are preceded by severe itching, called pruritus. The necrosis may require surgical débridement. To avoid this risk, hematologists recommend coadministration of LMWH or synthetic pentasaccharide (fondaparinux) with Coumadin for any patient suspected of protein C deficiency or known to have previously suffered skin necrosis until a satisfactory and stable international normalized ratio (INR) is reached (Chapter 43).

Activity levels of protein C and protein S remain below normal for 10 to 14 days after cessation of Coumadin therapy. Similarly, for several days after surgery or a thrombotic event, these proteins are diminished even if Coumadin has not been used.⁷⁰ Their activities are depressed in pregnancy, liver or renal disease, vitamin K deficiency, DIC, and with oral contraceptive use. Protein C and protein S assays therefore cannot be used to identify a congenital deficiency when they are employed within 14 days after thrombosis or after the cessation of Coumadin therapy, during pregnancy, or in the presence of DIC, liver disease, renal disease, vitamin K deficiency, or oral contraceptive use.⁷¹

Homozygous protein C or protein S deficiency results in *neonatal purpura fulminans*, a condition that is rapidly fatal when untreated. Treatment includes administration of factor concentrates and lifelong Coumadin therapy.

Protein C Assays

Functional assays detect both quantitative and qualitative protein C deficiencies.⁷² Either chromogenic or clot-based protein C activity assays are available. In the former, the laboratory professional first mixes the patient's plasma with Protac (Pentapharm, Inc., Basel, Switzerland), derived from the venom of the southern copperhead serpent *Agkistrodon contortrix*, which activates protein C. Subsequently, a chromogenic substrate is added, and its hydrolysis by the recently generated APC is measured by assessing the intensity of colored product, which is proportional to the activity of protein C (Figure 39-11). The assay detects abnormalities that affect the molecule's *proteolytic*

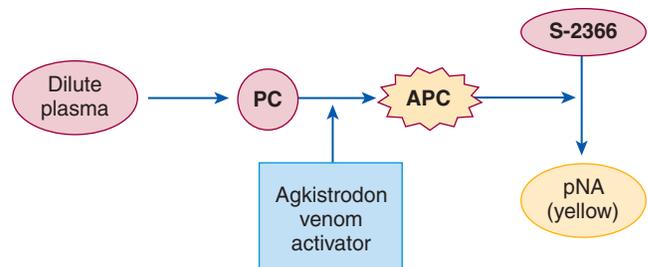


Figure 39-11 Chromogenic protein C (PC) assay. Patient plasma is pipetted into a reagent composed of *Agkistrodon contortrix* venom activator and chromogenic substrate (S-2366) specific to activated protein C (APC). The APC hydrolyzes the substrate to produce para-nitroaniline (pNA), a yellow product. The intensity of color is proportional to APC activity.

properties (active serine protease site) but misses those that affect protein C's phospholipid binding site or protein S binding site. In cases in which protein C chromogenic assays and immunoassays generate normal results but the clinical condition continues to indicate possible protein C deficiency, a clot-based protein C assay may detect abnormalities at these additional sites on the molecule.

The clot-based protein C assay is based on the ability of APC to prolong the PTT. The laboratory professional mixes plasma with protein C–depleted normal plasma to ensure normal levels of all factors except protein C. PTT reagent mixed with Protac and a heparin neutralizer is added, followed by calcium chloride, and the interval to clot formation is measured. Prolongation is proportional to plasma protein C activity. The clot-based protein C activity assay may be performed using an automated coagulation analyzer. Therapeutic heparin concentrations greater than 1 IU/mL consume the heparin neutralizer, prolong the PTT, and lead to overestimation of protein C. APC resistance, LA, and the presence of therapeutic *direct thrombin inhibitors* such as *argatroban* or *dabigatran* may prolong the PTT and falsely raise the protein C activity level in clot-based assays.

Enzyme immunoassay is used to measure protein C antigen when the functional activity is low and acquired causes have been ruled out. Microtiter plates coated with rabbit anti–human protein C antibody are used to capture test plasma protein C, and the concentration of antigen is measured by color development after the sequential addition of peroxidase-conjugated anti–human protein C and orthophenylenediamine substrate. The protein C antigen concentration assay detects most acquired deficiencies and quantitative congenital deficiencies, but it does not detect qualitative congenital abnormalities, which is why it is used only in response to an abnormally reduced protein C functional assay result.

Protein S Assays

As with testing for antithrombin and protein C deficiencies, protein S deficiency screening requires a functional assay. No chromogenic assay is available. The laboratory practitioner performs a clot-based assay by mixing the patient's plasma with protein S–depleted normal plasma to ensure normal levels of all factors other than protein S. APC and Russell viper venom are added in a buffer that contains a heparin neutralizer, followed by calcium chloride. The practitioner records the interval to clot formation. The more prolonged the test result, the higher the protein S activity (Figure 39-12). The clot-based protein S assay can be automated.

Therapeutic heparin levels greater than 1 IU/mL consume the heparin neutralizer and lead to overestimation of protein S activity. APC resistance, LA, and the presence of the therapeutic *direct thrombin inhibitors* argatroban or dabigatran may prolong the PTT and falsely raise the activity levels in clot-based protein S assays.⁷³ Coagulation factor VII activation may occur during prolonged refrigeration of plasma at 4° C to 10° C; this may cause underestimation of protein S activity and may affect prothrombin time-based coagulation factor assays such as factor V or factor X assays.⁷⁴

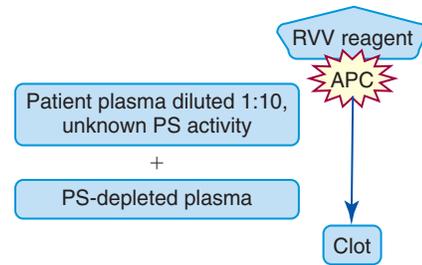


Figure 39-12 Clot-based protein S (PS) assay. Patient plasma is diluted and pipetted into a reagent composed of PS-depleted plasma. A second reagent is composed of Russell viper venom (RVV), which activates clotting at the level of factor X, and activated protein C (APC). The patient PS binds the reagent APC to prolong the clotting time. Clotting time is proportional to PS activity.

TABLE 39-6 Anticipated Protein S (PS) Test Results in Qualitative and Quantitative Deficiencies

Type of PS Deficiency	PS Activity	PS Free Antigen	PS Total Antigen	C4bBP
I Quantitative	<65%	<65%	<65%	Normal
II Qualitative	<65%	>65%	>65%	Normal
III Inflammation	<65%	<65%	>65%	Elevated

C4bBP, C4b-binding protein.

When there is clinical suspicion of primary protein S deficiency based on a low activity level, enzyme immunoassays are employed to measure both total and free protein S antigen. These assays detect most quantitative congenital deficiencies and aid in the diagnosis of qualitative type II congenital deficiencies characterized by normal antigen but decreased activity of protein S. In type III deficiency, the concentration of free antigen protein S and its activity, but not the total antigen, are reduced (Table 39-6). The concentration of plasma C4bBP, measured with an immunoassay available for research use only, aids in the classification of the type of protein S deficiency.

ARTERIAL THROMBOSIS PREDICTORS

Arterial thrombotic disease, including peripheral vascular occlusion, myocardial infarction (heart attack), and cerebrovascular ischemia (stroke), arises from atherosclerotic plaque. The traditional predictors of arterial thrombosis risk are elevated *total cholesterol* (TC) and *low-density lipoprotein cholesterol* (LDL-C), or an *elevated ratio of total cholesterol to high-density lipoprotein cholesterol* (TC:HDL-C) secondary to HDL-C deficiency. One third of primary cardiovascular and cerebrovascular events occur in patients whose lipid profiles are normal, however, and half of people with proven lipid risk factors never experience an arterial thrombotic event.⁷⁵

Researchers have sought additional arterial thrombosis predictors by performing prospective randomized studies of lipoprotein subtypes, fibrinolytic pathway components, and markers of inflammation. The results of these studies have led to the identification of potential markers of arterial thrombosis risk (Table 39-7), of which *homocysteine* and *high-sensitivity CRP*

TABLE 39-7 Markers of Arterial Thrombosis Risk

Marker	Reference Interval	Comments
High-sensitivity C-reactive protein	0.3–1.7 mg/L	Marker of inflammation; stable, reproducible
Fibrinogen	220–498 mg/dL	Chronic level >300 mg/dL increases thrombotic risk; inadequate reproducibility with numerous test platforms
Homocysteine	4.6–12.1 μ mol/L	Reference interval and predictive values vary with population; may be reduced with vitamin B ₆ , B ₁₂ , and folate supplementation
Total cholesterol (TC)	<200 mg/dL	Reproducible; some relationship with diet, exercise; risk prediction is partially dependent on inflammation
Ratio of total cholesterol to high-density lipoprotein cholesterol (TC : HDL-C)	<10	Reproducible; elevated ratio relates to diet, exercise; risk prediction is partially dependent on inflammation
Low-density lipoprotein cholesterol (LDL-C)	<130 mg/dL	Reproducible; may be significantly lowered with statin therapy
Lipoprotein (a)	2.2–49.4 mg/dL	Varies with race and age; lowered with statin therapy; inadequate reproducibility

have become a part of many institution's arterial thrombosis risk profiles. The role of platelets in arterial thrombosis is also under investigation.

C-Reactive Protein

CRP is a calcium-dependent pentameric ligand-binding member of the *pentraxin* family produced in the liver that circulates in plasma at a concentration below 0.55 mg/L. First described in 1930, CRP is an acute-phase reactant whose plasma concentration rises 1000-fold 6 to 8 hours after the onset of an inflammatory event such as an infection, trauma, or surgery. This rise remains stable over several days in vivo, and the protein is resistant to in vitro degradation.⁷⁶ Extremely high CRP levels are identified using one of several time-honored manual semiquantitative laboratory assays, all of which employ polyclonal anti-CRP antibodies that coats a suspension of visible latex particles. The test takes place on a slide or card. Laboratory professionals continue to use this simple and inexpensive assay to confirm inflammation and monitor the effectiveness of anti-inflammatory therapy.

A second CRP assay, high-sensitivity CRP (HSCRP), was developed in the late 1990s and is used to document modest but chronic CRP elevation. Both the manual HSCRP enzyme immunoassay and an automated latex microparticle immunoassay employ sensitive monoclonal antibodies that detect CRP at normal or slightly elevated concentrations. Chronic plasma HSCRP concentrations that remain at 1.5 mg/L or above indicate atherosclerosis secondary to low-grade inflammation that correlates to increased risk of myocardial infarction and stroke.⁷⁷ Consequently, HSCRP is a clinical measure, independent from the lipid profile, employed to predict cardiovascular or cerebrovascular disease (Table 39-8).⁷⁸ HSCRP may also be used in relationship with total cholesterol (Table 39-9) and the TC:HDL-C ratio (Table 39-10) to predict the risk of myocardial infarction. Laboratory professionals may also use HSCRP to monitor the anti-inflammatory effects of statins.⁷⁹⁻⁸²

Plasma Homocysteine

Homocysteine is a naturally occurring sulfur-containing amino acid formed in the metabolism of dietary *methionine*.^{83,84} The

TABLE 39-8 Relative Risk for Myocardial Infarction or Stroke at Four Levels of High-Sensitivity C-Reactive Protein (HSCRP) Independent of Lipid Levels

Quartile	HSCRP	Men	Women
1	≤0.55 mg/L	1.0	1.0
2	0.56–1.14 mg/L	1.8	2.9
3	1.15–2.10 mg/L	2.5	3.5
4	≥2.11 mg/L	2.9	5.5

TABLE 39-9 Relative Risk for Myocardial Infarction at Three Levels of High-Sensitivity C-Reactive Protein (HSCRP) Related to Total Cholesterol

HSCRP	TOTAL CHOLESTEROL		
	Low: ≤191 mg/dL	Medium: 192–223 mg/dL	High: ≥224 mg/dL
Low: ≤0.72 mg/L	1.0	1.4	2.3
Medium: 0.73–1.69 mg/L	1.2	1.5	4.3
High: ≥1.70 mg/L	1.1	2.3	5.3

TABLE 39-10 Relative Risk for Myocardial Infarction at Three Levels of High-Sensitivity C-Reactive Protein (HSCRP) Related to Ratio of Total Cholesterol to High-Density Lipoprotein Cholesterol (TC : HDL-C Ratio)

HSCRP	TC : HDL-C RATIO		
	Low: ≤3.78	Medium: 3.79–5.01	High: ≥5.02
Low: ≤0.72 mg/L	1.0	1.2	2.8
Medium: 0.73–1.69 mg/L	1.1	2.5	3.4
High: ≥1.70 mg/L	1.3	2.8	4.4

homocysteine concentration in plasma depends on adequate protein intake and adequate levels of vitamin B₆, vitamin B₁₂, and folate. Its concentration is regulated by three enzymes: cystathionine β-synthase, which converts homocysteine to *cystathionine* in the presence of vitamin B₆; 5,10-methylenetetrahydrofolate reductase (MTHFR), required for the remethylation of homocysteine to methionine in the folic acid cycle; and methionine synthase, which requires vitamin B₁₂. Folate, B₆, or B₁₂ deficiencies; common functional polymorphisms of the MTHFR gene; or an inherited deficiency of either cystathionine β-synthase or methionine synthase yields increased plasma homocysteine, *homocysteinemia*.⁸⁵

Clinical Significance of Homocysteinemia

Fasting homocysteinemia is an independent risk factor for arterial thrombosis, with relative risk ratios of 1.7 for coronary artery disease, 2.5 for cerebrovascular disease, and 6.8 for peripheral artery disease.^{86,87} Homozygosity for the MTHFR C677T mutation is associated with homocysteinemia but is not an independent thrombosis risk factor. Several theories link homocysteinemia with coronary artery disease, most citing damage to the endothelial cell.⁸⁸

Homocysteine Reference Range and Therapy

The reference ranges for homocysteine differ for men and women, and they rise with age, as shown in Table 39-11. No clinical outcomes studies have correlated homocysteine reduction with reductions in adverse arterial thrombosis events.

Fibrinogen Activity

Laboratory professionals measure fibrinogen using immunoassay, nephelometry, or the Clauss clot-based method to detect dysfibrinogenemia, hypofibrinogenemia, or afibrinogenemia (Chapter 42). The same assays may be used to detect chronic hyperfibrinogenemia. Fibrinogen concentration correlates with relative risk of myocardial infarction in asymptomatic persons or patients with angina pectoris, as shown in Table 39-12.⁸⁹ The relative risk triples from the first to the fifth quintiles, and even chronic high-normal levels predict increased risk. There also exists a direct correlation between fibrinogen and total cholesterol (Figure 39-13). High fibrinogen concentrations can be used to predict hypercholesterolemia and identify patients who are at high risk for new coronary events. In contrast, low normal fibrinogen levels are associated with low risk of cardiovascular events, even in people with high total cholesterol levels.

TABLE 39-11 Homocysteine Enzyme Immunoassay Reference Intervals

Population	Reference Interval (μmol/L)
Females ≤60 yr	4.6–12.1
Females >60 yr	6.6–14.1
Males ≤60 yr	5.0–15.6
Males >60 yr	7.0–17.6

TABLE 39-12 Relative Risk of Coronary Events According to Concentration of Fibrinogen*

Fibrinogen Concentration Quintile	Relative Risk of Coronary Event
1	1.0
2	1.89
3	2.33
4	2.56
5	2.89

*The relative risks are shown for each of five quintiles of subjects defined according to the concentrations of each fibrinogen from 1, the group with the lowest concentration, to 5, the group with the highest concentration. Relative risks have been adjusted for all confounding factors. The group with the lowest values serves as the reference group.

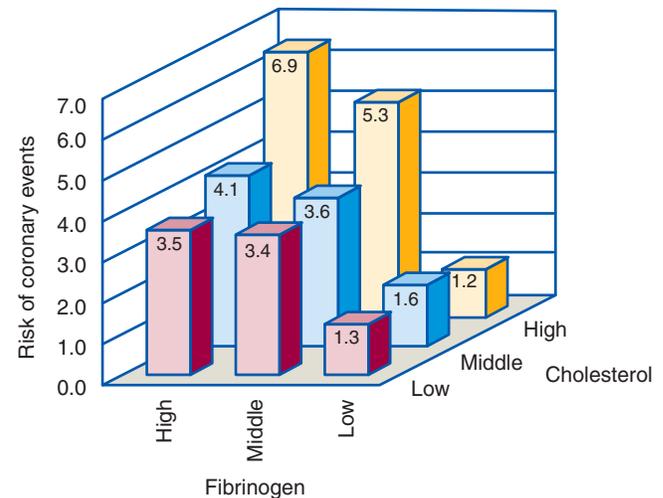


Figure 39-13 Coronary risk prediction by fibrinogen and cholesterol concentrations. Fibrinogen and total cholesterol synergistically predict coronary risk. Tertiles of fibrinogen concentration are shown in relation to tertiles of total cholesterol concentration with the relative risk of coronary events indicated for each combination.

Elevated fibrinogen supports coagulation and activates platelets by binding to their glycoprotein IIb/IIIa membrane receptors. Fibrinogen becomes integrated into atherothrombotic lesions and contributes to their thrombotic potential.

Although hyperfibrinogenemia predicts arterial thrombosis, the use of the fibrinogen assay for this purpose is limited. There are no independent therapeutic regimens that specifically lower fibrinogen, and no clinical trials suggest that fibrinogen reduction reverses the odds of thrombosis. Further, the various fibrinogen assay methods are not normalized. Nevertheless, statin therapy, smoking cessation, and exercise lower fibrinogen levels alongside LDL-C and total cholesterol levels, and the assay results parallel those for the other members of the risk prediction profile.

Lipoprotein (a)

Lipoprotein (a) is low-density lipoprotein that may be used to predict arterial thrombosis. The plasma level is measured by enzyme immunoassay, and its reference ranges are shown in Table 39-13. Although lipoprotein (a) concentrations are

TABLE 39-13 Normal Ranges for Lipoprotein (a) by Race and Sex

Race	Males	Females
African American	4.7–71.8 mg/dL	4.4–75.0 mg/dL
European American	2.2–49.4 mg/dL	2.1–57.3 mg/dL

higher in African Americans than in European Americans, the level is a stronger predictor of thrombosis in Caucasians.⁹⁰

Lipoprotein (a) may contribute to thrombosis by its antifibrinolytic property. The molecule competes with plasminogen for binding sites on newly formed fibrin polymer, decreasing the plasmin activity available for clot degradation. It also may contribute to the overall concentration of LDL-C. Levels can be lowered with statin drugs, as can LDL-C levels.

DISSEMINATED INTRAVASCULAR COAGULATION

DIC, also named *defibrination syndrome* or *consumption coagulopathy*, is the generalized activation of hemostasis secondary to a systemic disease. DIC involves all hemostatic systems: vascular intima, platelets, leukocytes, coagulation, coagulation control pathways, and fibrinolysis.⁹¹ In DIC, fibrin microthrombi partially occlude small vessels and consume platelets, coagulation factors, coagulation control proteins, and fibrinolytic enzymes. Fibrin/fibrinogen degradation products (FDPs, traditionally called *fibrin split products*, FSPs), including D-dimer, become elevated and interfere with normal fibrin formation.⁹² This combination of events sets loose a series of toxic and inflammatory processes.⁹³

DIC may be *acute and uncompensated*, with deficiencies of multiple hemostasis components, or *chronic*, with normal or even elevated clotting factor levels. In chronic DIC, liver coagulation factor production and bone marrow platelet production compensate for increased consumption.

Although DIC is a thrombotic process, the thrombi that form are small and ineffective, so systemic hemorrhage is often the first or most apparent sign. Acute DIC is often fatal and requires immediate medical intervention. The diagnosis relies heavily on the hemostasis laboratory, and medical laboratory professionals often perform a DIC profile under emergent circumstances.

Causes

Any disorder that contributes hemostatic molecules or promotes their endogenous secretion may cause DIC. Classifying and listing all the causes of DIC are impossible, but the major triggering mechanisms and examples of each are listed in Table 39-14.

The more acutely ill the patient, the more dangerous the symptoms. Chronic DIC may be associated with vascular tumors, tissue necrosis, liver disease, renal disease, chronic inflammation, use of prosthetic devices, and adenocarcinoma. The malignancies most associated with DIC are pancreatic, prostatic, ovarian, and lung cancers; multiple myeloma; and

TABLE 39-14 Conditions Associated with Disseminated Intravascular Coagulation Grouped by Mechanism

Mechanism	Examples of Conditions
Tissue factor is released into circulation through endothelial cell damage or monocyte activation	Physical trauma: crush or brain injuries, surgery Degradation of muscle; rhabdomyolysis Tissue ischemia; myocardial infarction Thermal injuries: burns or cold Adenocarcinoma
Exposure of subendothelial tissue factor during vasodilatation	Hypovolemic and hemorrhagic shock Malignant hypertension Asphyxia and hypoxia Heat stroke Vasculitis
Endotoxins that activate cytokines	Bacterial, protozoal, fungal, and viral infections, septicemia Toxic shock syndrome
Circulating immune complexes	Heparin-induced thrombocytopenia with thrombosis Drugs that trigger an immune response Acute hemolytic transfusion reactions Allergic reactions and anaphylaxis Bacterial and viral infections Graft rejection
Particulate matter from tissue injury	Eclampsia, preeclampsia, HELLP syndrome Retained dead fetus or missed abortion Amniotic fluid embolism Abruptio placentae Rupture of uterus Tubal pregnancy Fat embolism Heatstroke
Infusion of activated clotting factors	Activated prothrombin complex concentrate therapy
Secretion of proteolytic enzymes	Acute promyelocytic or myelomonocytic leukemia Bacterial, protozoal, fungal, and viral infections Pancreatitis
Toxins that trigger coagulation	Snake or spider envenomation Pancreatitis
Thrombotic disease or thrombogenic conditions	Thrombotic thrombocytopenic purpura, hemolytic uremic syndrome Pregnancy, postpartum period, estrogen therapy Deep vein thrombosis, pulmonary embolus Coagulation control system deficiencies Purpura fulminans, skin necrosis
Severe hypoxia or acidosis	Acute coagulopathy of trauma-shock Chronic inflammation Diabetes mellitus
Platelet activation	Vascular surgery, coronary artery bypass surgery Thrombocytosis, thrombocythemia, polycythemia Vascular tumors Vascular prostheses Aortic aneurysm

HELLP, Hemolysis, elevated liver enzymes, and low platelet count.

myeloproliferative diseases. Acute DIC is seen in association with obstetric emergencies, intravascular hemolysis, septicemia, viremia, burns, acute inflammation, crush injuries, dissecting aortic aneurysms, and cardiac disorders.

Pathophysiology

Triggering events may activate coagulation at any point in the pathway. When triggered, however, DIC proceeds in a predictable sequence of events. Circulating thrombin is the primary culprit because it activates platelets, activates coagulation proteins (positive feedback loops within the coagulation cascade), and catalyzes fibrin formation, of which the ensuing clots consume control proteins. The fibrinolytic system may become activated at the level of plasminogen or TPA subsequent to fibrin formation, and endothelial cells may become damaged, releasing coagulation-active substances. Finally, leukocytes—particularly monocytes—may be induced to secrete tissue factor by the cytokines released during inflammation.

Thrombin cleaves fibrinogen, creating fibrin monomers. In normal hemostasis, fibrin monomers spontaneously polymerize to form an insoluble gel. The polymer becomes strengthened through cross-linking, binding plasminogen as it forms. In DIC, a percentage of fibrin monomers fail to polymerize and circulate in plasma as *soluble fibrin monomers*. The monomers coat platelets and coagulation proteins, creating an anticoagulant effect (Figures 37-12 and 37-14).

Soluble fibrin monomers, fibrin polymer, and cross-linked fibrin all activate plasminogen. Normally, the active form of plasminogen—plasmin—acts locally to digest only the solid fibrin clot to which it is bound (Chapter 37). In DIC, plasmin circulates in the plasma and digests all forms of fibrinogen and fibrin.⁹⁴ Consequently, fibrin degradation products labeled X, Y, D, E, and D-dimer are detectable in the plasma in concentrations exceeding 20,000 ng/mL. D-dimer arises from cross-linked fibrin polymer, whereas the other fibrin degradation products may be produced from fibrinogen or fibrin monomers or polymers.⁹⁵

Platelets become enmeshed in the fibrin polymer or are exposed to thrombin; both events trigger platelet activation, which further drives the coagulation system and produces thrombocytopenia. At the same time, coagulation pathway control is lost as protein C, protein S, and antithrombin are consumed. The combination of thrombin activation, circulating plasmin, loss of control, and thrombocytopenia contributes to the overall hemorrhagic outcome of DIC.

Free plasmin digests factors V, VIII, IX, and XI, as well as other plasma proteins. Plasmin also may trigger complement, which leads to hemolysis, and the kinin system, which results in inflammation, hypotension, and shock. During fibrinolytic therapy, in amyloidosis, and sometimes in liver disease, plasminogen becomes activated independently of the coagulation pathway. This condition, called *systemic fibrinolysis* or *primary fibrinolysis*, produces laboratory-measurable fibrinogen and fibrin degradation products, including D-dimer, and prolonged prothrombin time (PT) and PTT with a normal platelet count.⁹⁶

Symptoms

The symptoms signaling DIC frequently are masked by the symptoms of the underlying disorder and may be chronic, acute, or even fulminant. Thrombosis in the microvasculature of major organs may produce symptoms of organ failure, such as renal function impairment, adult respiratory distress syndrome, and central nervous system manifestations. Skin, bone, and bone marrow necrosis may be seen. *Purpura fulminans* is seen in meningococemia, chickenpox, and spirochete infections.

Laboratory Diagnosis

DIC is a clinical diagnosis that requires laboratory confirmation (Chapter 42). The initial laboratory profile includes a platelet count, blood film examination, PT, PTT, D-dimer, and fibrinogen assay. Table 39-15 lists anticipated DIC results. Prolonged PT and PTT reflect coagulation factor consumption. Fibrinogen concentrations may decrease in DIC, but because fibrinogen is an acute phase reactant that rises in inflammation, the fibrinogen concentration alone provides little reliable information and may exceed 400 mg/dL. The peripheral blood film platelet estimate confirms thrombocytopenia in nearly all cases, and the presence of *schistocytes* helps establish the diagnosis of DIC in about 50% of cases.^{97,98} An elevated D-dimer level is essential to the diagnosis. D-dimer also may be elevated in inflammatory conditions, localized thrombosis, or renal disease in the absence of DIC; the PT, PTT, platelet count, blood film examination, and other laboratory assays must be performed alongside the D-dimer to rule out these disorders.

The D-dimer reference limit is typically 240 ng/mL, although the interval varies with location and technology. In 85% of DIC cases, D-dimer levels reach 10,000 to 20,000 ng/mL. A normal D-dimer assay result rules out DIC and rules out localized venous thromboembolic disease such as deep vein thrombosis or pulmonary emboli, in which levels rise to greater than 500 ng/mL but not to DIC levels.⁹⁹ D-dimer concentrations are typically elevated in inflammation, sickle cell crisis, pregnancy, and renal disease, so an abnormal result alone cannot be used to definitively diagnose venous thromboembolism.¹⁰⁰ The judicious use of the D-dimer assay reduces the requirement for

TABLE 39-15 Anticipated Results of Disseminated Intravascular Coagulation (DIC) Primary Laboratory Profile

Test	Reference Interval	Value in DIC
Platelet count	150,000–450,000/ μ L	<150,000/ μ L
Prothrombin time	11–14 sec	>14 sec
Partial thromboplastin time	25–35 sec	>35 sec
D-dimer	0–240 ng/mL	>240 ng/mL, often 10,000 to 20,000 ng/mL
Fibrinogen	220–498 mg/dL	<220 mg/dL, often higher, because fibrinogen is an acute phase reactant

invasive diagnostic tests such as pulmonary angiography when pulmonary embolism is suspected.¹⁰¹

Specialized Laboratory Tests That May Aid in Diagnosis

Table 39-16 lists specialized laboratory tests that may be used to diagnose and classify DIC in special circumstances. Results consistent with DIC and clinical comments are included. Many of these tests are available in acute care facilities, but they are not routinely applied to the diagnosis of DIC. Others are available only in tertiary care facilities and hemostasis reference laboratories.¹⁰²⁻¹⁰⁴

Protein C, protein S, and antithrombin typically are consumed in DIC, and their assay may contribute to the diagnosis. When thawed frozen plasma or antithrombin concentrate (Thrombate III) is used to treat DIC, these assays are useful in establishing the necessity for therapy and monitoring its effect.¹⁰⁵ Factor assays may clarify PT and PTT results. The thrombin time and the reptilase time also are sensitive DIC screens.

Tests of the fibrinolytic pathway include serum fibrin degradation products, now obsolete; chromogenic plasminogen activity assay; and TPA and PAI-1 immunoassays.¹⁰⁶ These tests are seldom offered in the acute care hemostasis laboratory because they require careful specimen management, but they may help in the diagnosis of primary fibrinolysis, which may occur after fibrinolytic therapy.

Treatment

To arrest DIC, the physician must diagnose and treat the underlying disorder. Surgery, anti-inflammatory agents, antibiotics, or obstetric procedures as appropriate may normalize hemostasis, particularly in chronic DIC. Supportive therapy, such as maintenance of fluid and electrolyte balance, always accompanies medical and surgical management.

In acute DIC, in which multiorgan failure from microthrombosis and bleeding threatens the life of the patient, heroic measures are necessary. Treatment falls into two categories: therapies

that slow the clotting process and therapies that replace missing platelets and coagulation factors.

UFH may be used for its antithrombotic properties to stop the uncontrolled activation of the coagulation cascade. Because UFH may aggravate bleeding, careful observation and support are required. Repeated chromogenic anti-factor Xa heparin assays may be necessary to control heparin dosage because in DIC the PTT is ineffective for monitoring heparin therapy.

Thawed frozen plasma provides all the necessary coagulation factors and replaces blood volume lost during acute DIC hemorrhage. Prothrombin complex concentrate (Proplex T complex, Baxter Healthcare Corporation, Deerfield, IL; or Kcentra, CSL Behring, King of Prussia, PA), fibrinogen concentrate (RiaSTAP, CSL Behring, King of Prussia, PA), and factor VIII concentrate (ADVATE, Baxter, Deerfield, IL) may be used in place of plasma, particularly if there is concern for transfusion-associated circulatory overload. Repeated measurements of fibrinogen, PT, and PTT are necessary to confirm the effectiveness of these therapeutics. Platelet transfusions are necessary if thrombocytopenia is severe. The effectiveness of platelet concentrate and platelet consumption are monitored with platelet counts (Chapters 15 and 16). Red blood cells are administered as necessary to treat the resulting anemia. Antifibrinolytic therapy is contraindicated, except in proven systemic fibrinolysis.^{107,108}

LOCALIZED THROMBOSIS MONITORS

In addition to D-dimer, several peptides and coagulation factor complexes are released into the plasma during coagulation. One complex, *thrombin-antithrombin complex* (TAT), and one peptide, *prothrombin fragment F 1+2* (PF 1+2 or PF 1.2), may be assayed as a means to detect and monitor localized venous or arterial thromboses. TAT and PF 1+2 immunoassays are sensitive and specific for thrombosis, which occurs in DIC, septicemia, eclampsia, pancreatitis, leukemia, liver disease, and

TABLE 39-16 Specialized Hemostasis Laboratory Assays Used to Diagnose and Classify Disseminated Intravascular Coagulation (DIC)

Assay	Value in DIC	Comments
Serum fibrin degradation products (FDP)	>10 $\mu\text{g}/\text{mL}$	Obsolete, replaced by quantitative D-dimer.
Soluble fibrin monomer	Positive	Hemagglutination assay provides valid measure of fibrin monomer. Avoid obsolete tests such as protamine sulfate solubility or ethanol gelation.
Thrombus precursor protein	>3.5 $\mu\text{g}/\text{mL}$	Immunoassay with no interference from fibrinogen or FDPs.
Protein C, protein S, and AT activity assays	<50%	Use to monitor therapy: plasma, AT concentrate, recombinant thrombomodulin.
Plasminogen, tissue plasminogen activator	Decreased	May be useful for analyzing systemic fibrinolysis. Specimen management protocol must be strictly observed.
Peripheral blood film exam	Anemia with schistocytes	Schistocytes (microangiopathic hemolytic anemia) are present in 50% of DIC cases; leukocytosis is common.
Localized thrombosis markers: prothrombin fragment 1+2, thrombin-antithrombin	Elevated	Most useful in diagnosis of localized thrombotic events, but may be used to monitor DIC therapy. Used in clinical trials.
Factor assays: II (prothrombin), V, VIII, X	<30%	Factors V and VIII rise in inflammation; assays may give misleading results.
Thrombin time, reptilase time	Prolonged	Fibrinogen levels <80 mg/dL, elevated FDPs, and soluble fibrin monomer all prolong thrombin time and reptilase time.

AT, Antithrombin; FDP, fibrin degradation product.

trauma.¹⁰⁸ These assays are of particular value in clinical trials of anticoagulants.

PF 1+2 is released from prothrombin at the time of its conversion to thrombin by the prothrombinase complex. It has a plasma half-life of 90 minutes and a reference range of 0.3 to 1.5 nmol/L. Elevated PF 1+2 may be seen in venous thromboembolism. Heparin or oral anticoagulant therapy reduces its plasma concentration.

The TAT covalent complex is formed when antithrombin neutralizes thrombin. This reaction is enhanced by the presence of heparin. TAT has a half-life of 3 minutes and a normal range of 0.5 to 5 ng/mL.

To avoid *in vitro* release or activation of PF 1+2 or TAT, plasma specimens are collected in 3.2% sodium citrate and are centrifuged and separated within minutes of collection; then the plasma is frozen until ready for assay.

HEPARIN-INDUCED THROMBOCYTOPENIA

Heparin-induced thrombocytopenia (HIT), also called *heparin-induced thrombocytopenia with thrombosis*, is an adverse effect of heparin treatment.

Cause and Clinical Significance

Between 1% and 5% of patients receiving unfractionated heparin (UFH) for more than 5 days develop an IgG antibody specific for heparin–platelet factor 4 complexes. In 30% to 50% of these cases, the immune complexes that are formed bind platelet Fc receptors, which leads to platelet activation, thrombocytopenia, and formation of microvascular thrombi.¹⁰⁹ HIT occurs with UFH administration at both prophylactic and therapeutic

dosages, although it is more frequent with therapeutic doses.¹¹⁰ Venous thrombosis predominates 5:1, but arterial thrombosis accounts for the most disturbing symptoms. Patients may develop pulmonary emboli, limb gangrene requiring amputation, stroke, and myocardial infarction. HIT is often a medical emergency, and the mortality rate is 20%.¹¹¹ LMWH also causes HIT. In most cases, HIT during LMWH therapy turns out to be a cross-reaction in a patient recently exposed to UFH; however, LMWH has been implicated as a primary cause at a rate of 1% of UFH-caused HIT. Likewise, protamine sulfate, a salmon sperm derivative that cardiac surgeons use to rapidly reduce UFH's anticoagulant may itself generate antibodies similar to anti-heparin-PF4 antibodies and cause thrombocytopenia and thrombosis symptoms indistinguishable from UFH-caused HIT.

Platelet Count

Patients receiving heparin must have platelet counts performed every other day. A platelet count decrease during heparin administration indicates HIT, but interpretation of the thrombocytopenia is confounded because 30% of patients receiving heparin develop an immediate, benign, and limited thrombocytopenia, sometimes called *HIT type I*.¹¹² This benign form of thrombocytopenia usually develops in 1 to 3 days, whereas thrombotic HIT, sometimes called *HIT type II*, develops after 5 days. However, thrombotic HIT may develop in 1 to 3 days in patients recently exposed to heparin. In HIT (HIT type II), the decrease in platelet count may exceed 40%, whereas in benign thrombocytopenia the decrease is relatively small; however, in both cases, the platelet count may remain within the normal range. The HIT diagnosis is made using the “4Ts” approach, provided in [Table 39-17](#).

TABLE 39-17 The “4Ts” Scoring System: Laboratory and Clinical Pretest Probability of Heparin-Induced Thrombocytopenia (HIT)

Indicator	SCORE		
	2	1	0
Acute thrombocytopenia	>50% decrease in platelet count to nadir of $\geq 20,000/\mu\text{L}$	30%–50% decrease in platelet count, >50% if directly resulting from surgery, or to nadir of 10,000–19,000/ μL	<30% decrease in platelet count, or to nadir of <10,000/ μL
Timing of platelet count decrease, thrombosis, or other sequelae of HIT (first day of heparin therapy is day 0)	Onset of decrease on days 5–10, or onset of decrease on day 1 if previous heparin exposure within past 5–30 days	Apparent decrease on days 5–10, but unclear due to missing platelet counts; or decrease after day 10; or decrease on day 1 if previous heparin exposure within past 31–100 days	Decrease at ≤ 4 days without recent heparin exposure
Thrombosis, skin lesions, acute system reaction	Proven new thrombosis or skin necrosis; acute systemic reaction after heparin exposure	Progressive, recurrent, or suspected thrombosis; erythematous skin lesions	None
Other causes for thrombocytopenia	No explanation for platelet count decrease is evident	Possible other cause is evident	Probable other cause is evident

From Crowther MA, Cook DJ, Albert M, et al: Canadian Critical Care Trials Group: the 4Ts scoring system for heparin-induced thrombocytopenia in medical-surgical intensive care unit patients, *J Crit Care* 25:287-293, 2010.

Maximum pretest probability score is 8; a score of 6 to 8 indicates high probability of HIT; 4 to 5, intermediate probability; 0 to 3, low probability.

The most immunizing heparin exposure is considered first; unfractionated heparin (UFH) received during cardiac surgery is more immunogenic than UFH or low-molecular-weight heparin received for acute coronary syndrome. The day the platelet count begins to fall is considered the day of onset of thrombocytopenia. It generally takes 1 to 3 days before an arbitrary threshold that defines thrombocytopenia, such as 150,000 platelets/ μL , is passed.

Laboratory Tests for HIT

Because at least 10% of hospitalized patients receive heparin, the acute care laboratory must provide a procedure to confirm HIT and differentiate it from benign thrombocytopenia. Most acute care laboratories provide a heparin-induced antibody immunoassay but seldom as a stat assay (Chapter 40).¹¹³ Owing to its sensitivity, the immunoassay result may be positive before clinical signs of HIT become evident. Further, the immunoassay result may be negative in a few HIT patients, possibly because peptides such as protamine sulfate may also form complexes with PF4. Microbial contamination, lipemia, and hemolysis may also invalidate immunoassay results. Conversely, patient specimen immune complexes or immunoglobulin aggregates may cause nonspecific binding and produce false-positive results.

Many laboratories provide aggregometry or lumiaggregometry methods to confirm HIT (Chapter 40).¹¹⁴ Aggregometry is technically demanding and is only 50% sensitive for HIT. Also technically demanding, but perhaps more sensitive, is washed platelet suspension lumiaggregometry. The reference method is the washed platelet ¹⁴C-serotonin release assay (serotonin release assay, SRA), provided by reference laboratories that possess radionuclide licenses.

Treatment

When heparin-induced antibodies are detected, the administration of UFH or LMWH is immediately discontinued, and the physician chooses an alternate form of anticoagulation. Complete cessation of anticoagulant therapy is risky because additional thrombotic events are likely to occur. Although LMWH causes HIT in only 1% of cases in which it is the sole anticoagulant, its use as a substitute for UFH is contraindicated owing to

its tendency to react with the existing antibody. Likewise, Coumadin is discouraged; it may precipitate a potentially severe skin necrosis if given in high bolus dosages, but more importantly its onset is too slow to be practical in this setting. Synthetic pentasaccharide (fondaparinux), which mimics heparin's antithrombin-binding sequence, has been shown in several small case series to be useful for the management of patients with suspected HIT.¹¹⁵

Recombinant bivalirudin (Angiomax, The Medicines Company, Parsippany, NJ) is a direct thrombin inhibitor modeled after leech saliva. Argatroban (Mitsubishi Pharma Corporation, Tokyo, Japan) is an amino acid analogue direct thrombin inhibitor. Both bind the active thrombin protease site, and, in HIT, physicians administer either argatroban or bivalirudin as a continuous infusion. Laboratory practitioners may monitor either using the PTT. The activated clotting time (ACT); the ecarin clotting time, which uses a reagent derived from *Echis carinatus* snake venom (Diagnostica Stago, Inc., Parsippany, NJ); or the plasma-diluted thrombin time (HEMOCLOT Thrombin, Aniara, Hyphen Biomed, West Chester, OH) may also be employed to monitor these direct thrombin inhibitors (Chapter 43).

CONCLUSION

The importance of laboratory diagnosis has become evident in all forms of thrombotic disease: chronic and acute, arterial and venous, primary and secondary, and acquired and congenital. The future may give us markers of endothelial cell disease, measures of leukocyte adhesion, and specific markers of inflammation and platelet activity that will further enable us to predict and prevent thrombosis.

SUMMARY

- Thrombosis is the most prevalent condition in developed countries and accounts for most illnesses and premature death.
- Thrombosis may be arterial, causing peripheral artery disease, heart disease, and stroke, or venous, causing deep vein thrombosis and pulmonary emboli.
- Most thrombosis occurs as a result of lifestyle habits and aging, but many thrombotic disorders are related to congenital risk factors.
- Thrombosis risk profiles may be offered to clinicians for screening purposes in high-risk populations.
- The main hemostasis predictors of arterial thrombotic disease are elevated levels of CRP (measured by high-sensitivity assay), homocysteine, fibrinogen, lipoprotein (a), and coagulation factors.
- The main hemostasis predictors of venous thromboembolic disease are APL antibodies, antithrombin, PC, and PS deficiency, FVL mutation, and prothrombin G20210A.
- APL antibody testing requires a series of essential hemostasis laboratory assays—clot-based tests and immunoassays.
- Antithrombin may be assayed using chromogenic substrate and enzyme immunoassay analyses.
- The tests for evaluating the protein C pathway include protein C and protein S activity and concentration, APC resistance, FVL assay, and C4bBP assay.
- The molecular test for the prothrombin G20210A mutation predicts the risk of venous thrombosis.
- DIC is a clinical diagnosis confirmed by a series of assays in the acute care facility.
- Chronic thrombosis may be identified using the PF 1+2, TAT complex, and quantitative D-dimer assays.
- The laboratory provides confirmatory tests for HIT with thrombosis.

Now that you have completed this chapter, go back and read again the case study at the beginning and respond to the questions presented.

REVIEW QUESTIONS

Answers can be found in the Appendix.

- What is the prevalence of venous thrombosis in the United States?
 - 0.01
 - 1 in 1000
 - 10% to 15%
 - 500,000 cases per year
- What is thrombophilia?
 - Predisposition to thrombosis secondary to a congenital or acquired disorder
 - Inappropriate triggering of the plasma coagulation system
 - A condition in which clots form uncontrollably
 - Inadequate fibrinolysis
- What acquired thrombosis risk factor is assessed in the hemostasis laboratory?
 - Smoking
 - Immobilization
 - Body mass index
 - Lupus anticoagulant
- Trousseau syndrome, a low-grade chronic DIC, is often associated with what type of disorder?
 - Renal disease
 - Hepatic disease
 - Adenocarcinoma
 - Chronic inflammation
- What is the most common heritable thrombosis risk factor in Caucasians?
 - APC resistance (factor V Leiden mutation)
 - Prothrombin G20210A mutation
 - Antithrombin deficiency
 - Protein S deficiency
- In most LA profiles, what screening test is primary because it detects LA with the fewest interferences?
 - Low-phospholipid PTT
 - DRVVT
 - KCT
 - PT
- A patient with venous thrombosis is tested for protein S deficiency. The protein S activity, antigen, and free antigen all are less than 65%, and the C4bBP level is normal. What type of deficiency is likely?
 - Type I
 - Type II
 - Type III
 - No deficiency is indicated, because the reference range includes 65%.
- An elevated level of what fibrinolytic system assay is associated with arterial thrombotic risk?
 - PAI-1
 - TPA
 - Factor VIIa
 - Factor XII
- How does lipoprotein (a) cause thrombosis?
 - It causes elevated factor VIII levels.
 - It coats the endothelial lining of arteries.
 - It substitutes for plasminogen or TPA in the forming clot.
 - It contributes additional phospholipid in vivo for formation of the Xase complex.
- What test may be used to *confirm* the presence of LA?
 - PT
 - Bethesda titer
 - Antinuclear antibody
 - PTT using high-phospholipid reagent
- What molecular test may be used to *confirm* APC resistance?
 - Prothrombin G20210A
 - MTHFR 1298
 - MTHFR 677
 - FVL
- What therapeutic agent may occasionally cause DIC?
 - Factor VIII
 - Factor VIIa
 - Antithrombin concentrate
 - Activated prothrombin complex concentrate
- Which is *not* a fibrinolysis control protein?
 - Thrombin-activatable fibrinolysis inhibitor
 - Plasminogen activator inhibitor-1
 - α 2-antiplasmin
 - D-dimer
- What is the most important application of the quantitative D-dimer test?
 - Diagnose primary fibrinolysis
 - Diagnose liver and renal disease
 - Rule out deep venous thrombosis
 - Diagnose acute myocardial infarction

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Thrombocytopenia and Thrombocytosis

Larry D. Brace

OUTLINE

Thrombocytopenia: Decrease in Circulating Platelets

*Impaired or Decreased Platelet Production
Increased Platelet Destruction
Abnormalities in Distribution or Dilution*

Thrombocytosis: Increase in Circulating Platelets

*Reactive (Secondary) Thrombocytosis
Thrombocytosis Associated with Myeloproliferative Disorders*

OBJECTIVES

After completion of this chapter, the reader will be able to:

1. Define thrombocytopenia and thrombocytosis, and state their associated platelet counts.
2. Compare and contrast the clinical symptoms of platelet disorders and clotting factor deficiencies.
3. Explain the primary pathophysiologic processes of thrombocytopenia.
4. Name and list the unique diagnostic features of at least four disorders included in congenital hypoplasia of the bone marrow and describe their inheritance patterns.
5. Differentiate between acute and chronic immune thrombocytopenia.
6. Describe the immunologic and nonimmunologic mechanisms by which drugs may induce thrombocytopenia.
7. Differentiate between neonatal isoimmune thrombocytopenia and neonatal autoimmune thrombocytopenia.
8. Explain the laboratory findings and pathophysiology associated with thrombotic thrombocytopenic purpura and hemolytic uremic syndrome.
9. Summarize the pathophysiology of thrombotic complications in heparin-induced thrombocytopenia and describe the sequence of treatment options.
10. Given clinical history and laboratory test results for patients with thrombocytopenia or thrombocytosis, suggest a diagnosis that is consistent with the information provided.

CASE STUDY

After studying the material in this chapter, the reader should be able to respond to the following case study:

An 18-month-old African-American girl sustained severe burns over 40% to 50% of her body, including both lower extremities. Within 1 month, she underwent a below-knee amputation of the left lower extremity. Over the next several years, she underwent skin-grafting surgeries, central venous line placement, and other burn-related surgeries. During these procedures, the patient was exposed to heparinized saline irrigation. Four years after the burn injury, thrombosis was noted in the right femoral artery during a grafting surgery. Unfractionated heparin was used during the surgery. Surgeons were unable to save the leg, and an above-knee amputation was necessary. At this time, hypercoagulability studies were ordered. Results were as follows:

	Patient Results	Reference Range
Protein C antigen	78%	70% to 137%
Protein S antigen	120%	63% to 156%
Antithrombin activity	111%	76% to 136%

The patient's results were normal on tests for the factor V Leiden and prothrombin G20210A mutations, and she was found not to have antiphospholipid antibody syndrome. Her platelet count had been decreasing steadily for 7 days before surgery but was still within the reference range.

1. Is the heparin used during the grafting surgeries significant in this patient's case?
2. What test should be ordered next?

Bleeding disorders resulting from platelet abnormalities, whether quantitative or qualitative, usually are manifested by bleeding into the skin or mucous membranes or both (mucocutaneous bleeding). Common presenting symptoms include petechiae, purpura, ecchymoses, epistaxis, and gingival bleeding. Similar findings also are seen in vascular disorders, but vascular disorders (e.g., Ehlers-Danlos syndrome, hereditary hemorrhagic telangiectasia) are relatively rare. In contrast, deep tissue bleeding, such as hematoma and hemarthrosis, is associated with clotting factor deficiencies.

THROMBOCYTOPENIA: DECREASE IN CIRCULATING PLATELETS

Although the reference range for the platelet count varies among laboratories, it is generally considered to be approximately 150,000 to 450,000/ μL (150,000 to 450,000/ mm^3 or 150 to 450 $\times 10^9/\text{L}$). Thrombocytopenia (platelet count of fewer than 100,000/ μL) is the most common cause of clinically important bleeding. True thrombocytopenia has to be differentiated from the thrombocytopenia artifact that can result from poorly prepared blood smears or automated cell counts when platelet clumping or platelet satellitosis are present (Chapters 15 and 16). The primary pathophysiologic processes that result in thrombocytopenia are decreased platelet production, accelerated platelet destruction, and abnormal platelet distribution (sequestration) (Box 40-1).

Small-vessel bleeding in the skin attributed to thrombocytopenia is manifested by hemorrhages of different sizes (Figure 40-1). *Petechiae* are small pinpoint hemorrhages about 1 mm in diameter, *purpura* are about 3 mm in diameter and generally round, and *ecchymoses* are 1 cm or larger and usually irregular in shape. Ecchymosis corresponds with the lay term *bruise*. Other conditions such as defective platelet function, vascular fragility, and trauma contribute to the hemorrhagic state.

Clinical bleeding varies and often is not closely correlated with the platelet count. It is unusual for clinical bleeding to occur when the platelet count is greater than 50,000/ μL , but the risk of clinical bleeding increases progressively as the platelet count decreases from 50,000/ μL . Patients with platelet counts of 20,000/ μL or sometimes lower may have little or no bleeding symptoms. In general, patients with platelet counts of fewer than 10,000/ μL are considered to be at high risk for a serious hemorrhagic episode.

Impaired or Decreased Platelet Production

Abnormalities in platelet production may be divided into two categories: One type is associated with megakaryocyte hypoplasia in the bone marrow, and the other type is associated with ineffective thrombopoiesis, as may be seen in disordered proliferation of megakaryocytes.

Inherited Thrombocytopenia/Congenital Hypoplasia

It is increasingly apparent that most inherited thrombocytopenias can be linked to fairly specific chromosomal abnormalities

BOX 40-1 Classification of Thrombocytopenia

Impaired or Decreased Production of Platelets

Congenital

- May-Hegglin anomaly
- Bernard-Soulier syndrome
- Fechtner syndrome
- Sebastian syndrome
- Epstein syndrome
- Montreal platelet syndrome
- Fanconi anemia
- Wiskott-Aldrich syndrome
- Thrombocytopenia with absent radii (TAR) syndrome
- Congenital amegakaryocytic thrombocytopenia
- Autosomal dominant and X-linked thrombocytopenia

Neonatal

Acquired

- Viral
- Drug induced

Increased Platelet Destruction

Immune

- Acute and chronic immune thrombocytopenic purpura

Drug induced: immunologic

- Heparin-induced thrombocytopenia
- Neonatal alloimmune (isoimmune neonatal) thrombocytopenia
- Neonatal autoimmune thrombocytopenia
- Posttransfusion isoimmune thrombocytopenia
- Secondary autoimmune thrombocytopenia

Nonimmune

- Thrombocytopenia in pregnancy and preeclampsia
- Human immunodeficiency virus infection
- Hemolytic disease of the newborn
- Thrombotic thrombocytopenia purpura
- Disseminated intravascular coagulation
- Hemolytic uremic syndrome
- Drug induced

Abnormalities of Distribution or Dilution

- Splenic sequestration
- Kasabach-Merritt syndrome
- Hypothermia
- Loss of platelets: massive blood transfusions, extracorporeal circulation



Figure 40-1 A, Petechiae, B, purpura, and C, ecchymoses indicate the various patterns of systemic (mucocutaneous) hemorrhage. (Fig. 40-1A and 40-1C from Gary P. Williams, MD, University of Wisconsin Clinical Science Center, Madison, WI.) (Fig. 40-1B from Kitchens CS, Alving BM, Kessler CM: *Consultative hemostasis and thrombosis*, Philadelphia, 2002, Saunders.)

or specific genetic defects. [Table 40-1](#) provides a list of inherited thrombocytopenias associated with specific gene and chromosomal abnormalities, mode of inheritance, and associated features.

Lack of adequate bone marrow megakaryocytes (megakaryocytic hypoplasia) is seen in a wide variety of congenital disorders, including Fanconi anemia (pancytopenia), thrombocytopenia with absent radius (TAR) syndrome, Wiskott-Aldrich syndrome, Bernard-Soulier syndrome, May-Hegglin anomaly, and several other less common disorders. Although thrombocytopenia is a feature of Bernard-Soulier syndrome and Wiskott-Aldrich syndrome, the primary abnormality in these disorders is a qualitative defect, and these disorders are discussed in Chapter 41.

May-Hegglin Anomaly. May-Hegglin anomaly is a rare autosomal dominant disorder; the exact frequency is unknown. Large platelets (20 μm in diameter) are present on the peripheral blood film, and Döhle-like bodies are present in neutrophils ([Figure 40-2](#)) and occasionally in monocytes. Other than the increase in size, platelet morphology is normal. Thrombocytopenia is present in about one third to one half of affected patients. Platelet function in response to platelet-activating agents is usually normal. In some patients, megakaryocytes are increased in number and have abnormal ultrastructure. Mutations in the *MYH9* gene that encodes for nonmuscle myosin heavy chain (a cytoskeletal protein in platelets) have been reported.¹ This mutation may be responsible for the abnormal size of platelets in this disorder. Most patients are asymptomatic unless severe thrombocytopenia is present, but bleeding times may be prolonged in some patients in the absence of bleeding complications ([Table 40-1](#)).

Three other disorders involving mutations of the *MYH9* gene have been reported: Sebastian syndrome, Fechtner syndrome, and Epstein syndrome.² Sebastian syndrome is inherited as an autosomal dominant disorder characterized by large platelets, thrombocytopenia, and granulocytic inclusions. Similar abnormalities are observed in Fechtner syndrome and are accompanied by deafness, cataracts, and nephritis. In Epstein syndrome, large platelets are associated with deafness, ocular problems, and glomerular nephritis.³ These disorders are discussed in more detail in Chapter 41.

TAR Syndrome. TAR syndrome is a rare autosomal recessive disorder characterized by severe neonatal thrombocytopenia and congenital absence or extreme hypoplasia of the radial bones of the forearms with absent, short, or malformed ulnae and other orthopedic abnormalities. TAR syndrome is associated with a mutation in the *RBM8A* gene located on the long arm of chromosome 1 or a 200 Kb deletion involving the *RBM8A* gene (1q21.1). TAR can result from two deletions of the *RBM8A* gene, two mutations of the *RBM8A* gene, or, most commonly, a combination of the two ([Table 40-1](#)). In addition to bony abnormalities, patients tend to have cardiac lesions and a high incidence of transient leukemoid reactions with elevated white blood cell (WBC) counts (sometimes with counts above 100,000/ μL) in 90% of patients.⁴ Platelet counts

TABLE 40-1 List of Inherited Thrombocytopenias

Disease (abbreviation, OMIM entry)	Frequency*/Spontaneous Bleeding	Inheritance	Gene (chromosome localization)	Other Features
SYNDROMIC FORMS				
Wiskott-Aldrich syndrome (WAS, 301000)	++++/yes	XL	<i>WAS</i> (Xp11)	Severe immunodeficiency leading to death in infancy; small platelets
X-linked thrombocytopenia (XLT, 313900)				Mild immunodeficiency; small platelets
<i>MYH9</i> -related disease (<i>MYH9</i> -RD, nd)	++++/no	AD	<i>MYH9</i> (22q12-13)	Cataracts, nephropathy and/or deafness; giant platelets; also non-syndromic
Paris-Trousseau thrombocytopenia (TCPT, 188025/600588), Jacobsen syndrome (JBS, 147791)	++++/yes	AD	Large deletion (11q23-ter)	Cardiac and facial defects, developmental delay and/or other defects; large platelets
Thrombocytopenia with absent radii (TAR, 274000)	++++/yes	AR	<i>RBM8A</i> (1q21.1)	Platelet count tends to rise and often normalizes in adulthood; reduced megakaryocytes; normal-sized platelets. Bilateral radial aplasia and/or other malformations
<i>GATA1</i> -related disease (<i>GATA1</i> -RD) (Dyserythropoietic anemia with thrombocytopenia-nd, 300367-X-linked thrombocytopenia with thalassemia-XLTT, 314050)	++yes	XL	<i>GATA1</i> (Xp11)	Hemolytic anemia, possible unbalanced globin chain synthesis, possible congenital erythropoietic porphyria; large platelets
Congenital thrombocytopenia with radio-ulnar synostosis (CTRUS, 605432)	+/yes	AD	<i>HOXA11</i> (7p15-14)	Radio-ulnar synostosis and/or other defects; possible evolution into aplastic anemia; normal sized platelets
Thrombocytopenia associated with sitosterolemia (STSL, 210250)	+/no	AR	<i>ABCG5</i> , <i>ABCG8</i> (2p21)	Anemia, tendon xanthomas, atherosclerosis; large platelets; also non-syndromic
<i>FLNA</i> -related thrombocytopenia (<i>FLNA</i> -RT, nd)	+/yes	XL	<i>FLNA</i> (Xq28)	Periventricular nodular heterotopia (MIM 300049); large platelets; also non-syndromic
NON-SYNDROMIC FORMS				
Bernard-Soulier syndrome (BSS, 231200)				
Biallelic	++++/yes	AR	<i>GP1BA</i> (17p13),	Giant platelets
Monoallelic	+++/no	AD	<i>GP1BB</i> (22q11), <i>GP9</i> (3q21)	Large platelets
Congenital amegakaryocytic thrombocytopenia (CAMT, 604498)	+++/yes	AR	<i>MPL</i> (1p34)	Always evolves into bone marrow aplasia in infancy; normal-sized platelets
Familial platelet disorder and predisposition to acute myelogenous leukemia (FPD/AML, 601399)	++/no	AD	<i>RUNX1</i> (21q22)	High risk of developing leukemia or MDS; normal-sized platelets
Gray platelet syndrome (GPS, 139090)	++/yes	AR	<i>NBEAL2</i> (3p21.1)	High risk of developing evolutive myelofibrosis and splenomegaly; giant platelets
<i>ANKRD26</i> -related thrombocytopenia (<i>THC2</i> , 313900)	++/no	AD	<i>ANKRD26</i> (10p11-12)	May be at risk of leukemia; normal-sized platelets
<i>ITGA2B/ITGB3</i> -related thrombocytopenia (<i>ITGA2B/ITGB3</i> -RT, nd)	+/no	AD	<i>ITGA2B</i> (17q21.31), <i>ITGB3</i> (17q21.32)	Large platelets
<i>TUBB1</i> -related thrombocytopenia (<i>TUBB1</i> -RT, nd)	+/no	AD	<i>TUBB1</i> (6p21.3)	Giant platelets
<i>CYCS</i> -related thrombocytopenia (<i>THC4</i> , 612004)	+/no	AD	<i>CYCS</i> (7p15.3)	Normal-sized platelets

AD, Autosomal dominant; AR, autosomal recessive; MDS, myelodysplastic syndrome; nd, not defined; OMIM, Online Mendelian Inheritance in Man; XL, X-linked. Some forms are categorized as both syndromic and non-syndromic. *++++, >100 families; +++, >50 families; ++ >10 families; +, <10 families. From: Balduini CL, Savoia A, Seri M. Inherited thrombocytopenias frequently diagnosed in adults. *J Thromb Haemost* 2013;11:1006-1019.



Figure 40-2 Döhle body in segmented neutrophil and giant platelets associated with May-Hegglin anomaly (peripheral blood, $\times 1000$). (From Carr JH, Rodak BF: *Clinical hematology atlas*, ed 4, Philadelphia, 2013, Saunders.)

are usually 10,000 to 30,000/ μL in infancy. Interestingly, platelet counts usually increase over time, with normal levels often achieved within 1 year of birth.

Fanconi Anemia. Fanconi anemia is also associated with thrombocytopenia, although other abnormalities are extensive, including bony abnormalities, abnormalities of visceral organs, and pancytopenia. Chapter 22 contains a more detailed description.

Congenital Amegakaryocytic Thrombocytopenia

Congenital amegakaryocytic thrombocytopenia is an autosomal recessive disorder reflecting bone marrow failure.⁵ Affected infants usually have platelet counts of fewer than 20,000/ μL at birth, petechiae and evidence of bleeding at or shortly after birth, and frequent physical anomalies. About half of the infants develop aplastic anemia in the first year of life, and there are reports of myelodysplasia and leukemia later in childhood. Allogeneic stem cell transplantation is considered curative for infants with clinically severe disease or aplasia.⁶ This disorder is caused by mutations in the *MPL* gene on chromosome 1 (1p34), resulting in complete loss of thrombopoietin receptor function (Table 40-1). This loss of function results in reduced megakaryocyte progenitors and high thrombopoietin levels.⁷

Autosomal Dominant Thrombocytopenia

Autosomal dominant thrombocytopenia has been mapped to a mutation(s) in the *ANKRD26* gene on the short arm of chromosome 10 (10p11-12). Mutations in this gene appear to lead to incomplete megakaryocyte differentiation and the resultant thrombocytopenia. Platelet morphology and size are usually normal. Until recently, autosomal dominant thrombocytopenia was considered a very rare disorder. However, *ANKRD26* mutations have recently been found in 21 of 210 thrombocytopenic pedigrees. This indicates that *ANKRD26* mutations may be responsible for approximately 10% of inherited

thrombocytopenias and is a relatively frequent form of autosomal dominant thrombocytopenia.⁸ Bleeding in these patients is usually absent or mild, and platelet function is usually normal (Table 40-1).^{9,10}

X-Linked Thrombocytopenia

X-linked thrombocytopenia can result from mutations in the *WAS* (Wiskott-Aldrich syndrome) gene on the X chromosome (Xp11) or mutations in the *GATA1* gene, also on the X chromosome at Xp11.¹¹⁻¹³ X-linked thrombocytopenias range from mild thrombocytopenia and small platelets and absent or mild bleeding to macrothrombocytopenia with severe bleeding (Table 40-1).

Other Inherited Thrombocytopenias

In addition to the inherited thrombocytopenias discussed above, there are several others that are due to gene mutations, including *HOXA11*, *ABCG5* and *ABCG8*, *FLNA*, *RUNX1*, *ITGA2B*, *ITGB3*, *TUBB1*, and *CYCS* (Table 40-1).

Neonatal Thrombocytopenia

Neonatal thrombocytopenia (platelet count $< 150,000/\mu\text{L}$) is present in 1% to 5% of infants at birth. The causes of neonatal thrombocytopenia are numerous as illustrated in Table 40-2. In 75% of cases, the thrombocytopenia is present at or within 72 hours of birth. Only a minority of these patients have immunologic disorders or coagulopathy causing thrombocytopenia.

Causes of neonatal thrombocytopenia include infection with *Toxoplasma*, rubella, cytomegalovirus (CMV), herpes (TORCH), and human immunodeficiency virus (HIV), and in utero exposure to certain drugs, particularly chlorothiazide diuretics and the oral hypoglycemic tolbutamide and other agents. TORCH infections cause thrombocytopenia with characteristically small platelets. CMV is the most common infectious agent causing congenital thrombocytopenia, with an overall incidence of 0.5% to 1% of all births,¹⁴ but only 10% to 15% of infected infants have symptomatic disease,¹⁵ which suggests that the incidence of significant neonatal thrombocytopenia caused by CMV is about 1 in 1000 infants. Although the mechanism of thrombocytopenia is not well understood, reports suggest that CMV inhibits megakaryocytes and their precursors, which results in impaired platelet production.¹⁶ About 1 in 1000 to 1 in 3000 infants are affected by congenital toxoplasmosis. About 40% of such infants develop thrombocytopenia.¹⁷ While persistent thrombocytopenia is a prominent feature in infants with congenital rubella syndrome, it is now rare in countries with organized immunization programs.^{18,19} Thrombocytopenia also is a feature of maternal transmission of HIV to the neonate and is a sign of intermediate to severe disease.²⁰

Maternal ingestion of chlorothiazide diuretics or tolbutamide can have a direct cytotoxic effect on the fetal marrow megakaryocytes. Thrombocytopenia may be severe, with platelet counts of 70,000/ μL and sometimes lower. Bone marrow examination reveals a marked decrease or absence of megakaryocytes. The thrombocytopenia develops gradually and is slow to regress when the drug is stopped. Recovery usually occurs within a few weeks after birth.^{10,21,22}

TABLE 40-2 Classification of Fetal and Neonatal Thrombocytopenias

Fetal	Alloimmune	
	Congenital infection (e.g., CMV, toxoplasma, rubella, HIV)	
	Aneuploidy (e.g., trisomies 18, 13, 21, or triploidy)	
	Autoimmune (e.g., ITP, SLE)	
	Severe Rh hemolytic disease	
	Congenital/inherited (e.g., Wiskott-Aldrich syndrome)	
Early onset neonatal (<72 hours)	Placental insufficiency (e.g., preeclampsia, IUGR, diabetes)	
	Perinatal asphyxia	
	Perinatal infection (e.g., <i>E. coli</i> , group B streptococcus, <i>Haemophilus influenzae</i>)	
	DIC	
	Alloimmune	
	Autoimmune (e.g., ITP, SLE)	
	Congenital infection (e.g., CMV, toxoplasma, rubella, HIV)	
	Thrombosis (e.g., aortic, renal vein)	
	Bone marrow replacement (e.g., congenital leukemia)	
	Kasabach-Merritt syndrome	
	Metabolic disease (e.g., propionic and methylmalonic acidemia)	
	Congenital/inherited (e.g., TAR, CAMT)	
	Late onset neonatal (>72 hours)	Late onset sepsis
		NEC
Congenital infection (e.g., CMV, toxoplasma, rubella, HIV)		
Autoimmune		
Kasabach-Merritt syndrome		
Metabolic disease (e.g., propionic and methylmalonic acidemia)		
Congenital/inherited (e.g., TAR, CAMT)		

CAMT, Congenital amegakaryocytic thrombocytopenia; *CMV*, cytomegalovirus; *DIC*, disseminated intravascular coagulation; *ITP*, immune thrombocytopenic purpura; *IUGR*, intrauterine growth restriction; *NEC*, necrotizing enterocolitis; *SLE*, systemic lupus erythematosus; *TAR*, thrombocytopenia with absent radii.

From: Roberts I, Murray NA. Neonatal thrombocytopenia: causes and management. *Arch Dis Child Fetal Neonatal Ed* 2003;88:F359-F364.

While infectious agents and certain drugs are well-known causes of neonatal thrombocytopenia, the overwhelming cause is impaired production. Most patients are preterm neonates born after pregnancies complicated by placental insufficiency and/or fetal hypoxia (preeclampsia and intrauterine growth restriction). These neonates have early-onset thrombocytopenia and impaired megakaryopoiesis in spite of increased levels of thrombopoietin (Table 40-2).

Increased platelet consumption/sequestration is another mechanism of neonatal thrombocytopenia accounting for approximately 2% to 25% of neonatal thrombocytopenia. Of these, 15% to 20% result from transplacental passage of maternal alloantibodies and autoantibodies (see neonatal alloimmune thrombocytopenia and neonatal autoimmune

thrombocytopenia later in this chapter). Another 10% to 15% of cases are due to disseminated intravascular coagulation (DIC), almost always in very ill infants, particularly those with perinatal asphyxia or infections. Other examples include thrombosis, platelet activation, or immobilization at sites of inflammation (e.g., necrotizing enterocolitis). In very sick infants, splenic sequestration may be a contributing factor to thrombocytopenia.

Inherited thrombocytopenic syndromes are increasingly being recognized as causes of neonatal thrombocytopenia (Tables 40-1 and 40-2). Although considered to be rare, they may be more common than once believed.

Acquired (Drug-Induced) Hypoplasia

A wide array of chemotherapeutic agents used for the treatment of hematologic and nonhematologic malignancies suppress bone marrow megakaryocyte production and the production of other hematopoietic cells. Examples include the commonly used agents methotrexate, busulfan, cytosine arabinoside, cyclophosphamide, and cisplatin. The resulting thrombocytopenia may lead to hemorrhage, and the platelet count should be monitored closely. Drug-induced thrombocytopenia is often the dose-limiting factor for many chemotherapeutic agents. Recombinant interleukin-11 has been approved for treatment of chemotherapy-induced thrombocytopenia, and thrombopoietin may prove to be useful for this purpose.²³⁻²⁵ Zidovudine (used for the treatment of HIV infection) is also known to cause myelotoxicity and severe thrombocytopenia.²⁶

Several drugs specifically affect megakaryocytopoiesis without significantly affecting other marrow elements. Anagrelide is one such agent, although its mechanism of action is unknown. This characteristic has made anagrelide useful for treating the thrombocytosis of patients with essential thrombocythemia and other myeloproliferative disorders.²⁷

Ingestion of ethanol for long periods (months to years) may result in persistent severe thrombocytopenia. Although the mechanism is unknown, studies indicate that alcohol can inhibit megakaryocytopoiesis in some individuals. Mild thrombocytopenia is a common finding in alcoholic patients, but other causes unrelated to ethanol use, such as portal hypertension, splenomegaly, and folic acid deficiency, should be excluded. The platelet count usually returns to normal within a few weeks of alcohol withdrawal, but thrombocytopenia may persist for longer periods. A transient rebound thrombocytosis may develop when alcohol ingestion is stopped.¹⁰

Interferon therapy commonly causes mild to moderate thrombocytopenia, although under certain circumstances, the thrombocytopenia can be severe and life-threatening. Interferon- α and interferon- γ inhibit stem cell differentiation and proliferation in the bone marrow, but the mechanism of action is unclear.²⁸

Thrombocytopenia presumably caused by megakaryocyte suppression also has been reported to follow the administration of large doses of estrogen or estrogenic drugs such as diethylstilbestrol. Other drugs, such as certain antibacterial agents (e.g., chloramphenicol), tranquilizers, and anticonvulsants, also have

been associated with thrombocytopenia caused by bone marrow suppression.²⁹⁻³¹

Ineffective Thrombopoiesis

Thrombocytopenia is a usual feature of the megaloblastic anemias (pernicious anemia, folic acid deficiency, and vitamin B₁₂ deficiency). Quantitative studies indicate that, as with erythrocyte production in these disorders, platelet production is ineffective. Although the bone marrow generally contains an increase in the number of megakaryocytes, the total number of platelets released into the circulation is decreased. Thrombocytopenia is caused by impaired DNA synthesis, and the bone marrow may contain grossly abnormal megakaryocytes with deformed, dumbbell-shaped nuclei, sometimes in large numbers. Stained peripheral blood films reveal large platelets that may have a decreased survival time and may have abnormal function. Thrombocytopenia is usually mild, and there is evidence of increased platelet destruction. Patients typically respond within 1 to 2 weeks to vitamin replacement.^{22,32-34}

Miscellaneous Conditions

Viruses are known to cause thrombocytopenia by acting on megakaryocytes or circulating platelets, either directly or in the form of viral antigen-antibody complexes. Live measles vaccine can cause degenerative vacuolization of megakaryocytes 6 to 8 days after vaccination. Some viruses interact readily with platelets by means of specific platelet receptors. Other viruses associated with thrombocytopenia include CMV, varicella-zoster virus, rubella virus, Epstein-Barr virus (which causes infectious mononucleosis), and the virus that causes Thai hemorrhagic fever.¹⁰

Certain bacterial infections commonly are associated with the development of thrombocytopenia. This may be the result of toxins of bacterial origin, direct interactions between bacteria and platelets in the circulation, or extensive damage to the endothelium, as in meningococcemia. Many cases of thrombocytopenia in childhood result from infection. Purpura may occur in many infectious diseases in the absence of thrombocytopenia, presumably because of vascular damage (Chapter 41).^{10,35}

A common cause of unexplained thrombocytopenia is infiltration of the bone marrow by malignant cells with a progressive decrease in marrow megakaryocytes as the abnormal cells replace normal marrow elements. Inhibitors of thrombopoiesis may be produced by these abnormal cells and may help to account for the thrombocytopenia associated with conditions such as myeloma, lymphoma, metastatic cancer, and myelofibrosis.^{22,32,36}

Increased Platelet Destruction

Thrombocytopenia as a result of increased platelet destruction can be separated into two categories: increased platelet destruction caused by immunologic responses and increased destruction caused by mechanical damage or consumption or both. Regardless of the process, increased production is required to maintain a normal platelet count, and the patient becomes

thrombocytopenic only when production capacity is no longer adequate to compensate for the increased rate of destruction.

Immune Mechanisms of Platelet Destruction

Immune (Idiopathic) Thrombocytopenic Purpura: Acute and Chronic. The term *idiopathic thrombocytopenic purpura (ITP)* was used previously to describe cases of thrombocytopenia arising without apparent cause or underlying disease state. Although the acronym for the disorder remains the same, the word *idiopathic* has been replaced by *immune* because of the realization that acute and chronic ITP are immunologically mediated.

Acute ITP. This is primarily a disorder of children, although a similar condition is seen occasionally in adults. The disorder is characterized by the abrupt onset of bruising, petechiae, and sometimes mucosal bleeding (e.g., epistaxis) in a previously healthy child. The primary hematologic feature is thrombocytopenia, which frequently occurs 1 to 3 weeks after an infection.

The infection is most often a nonspecific upper respiratory tract or gastrointestinal tract viral infection, but acute ITP also may occur after rubella, rubeola, chickenpox, or other viral illnesses and may follow live virus vaccination.³⁷ The incidence of acute ITP is estimated to be 4 in 100,000 children, with a peak frequency in children between 2 and 5 years of age. There is no sex predilection. In about 10% to 15% of the children initially thought to have acute ITP, the thrombocytopenia persists for 6 months or longer, and these children are reclassified as having chronic ITP.³⁸ The observation that acute ITP often follows a viral illness suggests that some children produce antibodies and immune complexes against viral antigens and that platelet destruction may result from the binding of these antibodies or immune complexes to the platelet surface.

The diagnosis of acute ITP in a child with severe thrombocytopenia almost always can be made without a bone marrow examination. If the child has recent onset of bleeding signs and symptoms, otherwise normal results on complete blood count (for all red and white blood cell parameters and cell morphology), and normal findings on physical examination (except for signs of bleeding), there is a high likelihood that the child has ITP. In addition, if the bleeding symptoms develop suddenly and there is no family history of hemorrhagic abnormalities or thrombocytopenia, the diagnosis of ITP is almost certain. There is, at present, no specific test that is diagnostic of acute or chronic ITP.

In mild cases of acute ITP, patients may have only scattered petechiae. In most cases of acute ITP, however, patients develop fairly extensive petechiae and some ecchymoses and may have hematuria or epistaxis or both. About 3% to 4% of acute ITP cases are considered severe, and typically generalized purpura is present, often accompanied by gastrointestinal bleeding, hematuria, mucous membrane bleeding, and retinal hemorrhage. Of patients with severe disease, 25% to 50% are considered to be at risk for intracranial hemorrhage, which is the primary complication that contributes to the overall 1% to 2% mortality rate for patients with acute ITP.³⁸ Most patients with life-threatening hemorrhage have a platelet count of less than

4000/ μL .³⁹ Hemorrhage is rarely experienced by patients whose platelet count exceeds 10,000/ μL .

Most patients with acute ITP recover with or without treatment in about 3 weeks, although for some, recovery may take 6 months. In a few children, recurrent episodes of acute ITP are occasionally seen after complete recovery from the first episode.⁴⁰ Most patients with acute ITP have relatively mild symptoms, and no treatment is needed. The most severe cases may need to be treated, however, and intravenous immunoglobulin (IVIG), platelet transfusions, and splenectomy (or some combination of these) seem to offer the most immediate benefit.^{37,38}

Chronic ITP. This disorder can be found in patients of any age, although most cases occur in patients between the ages of 20 and 50 years. Females with this disorder outnumber males 2:1 to 3:1, with the highest incidence in women between 20 and 40 years of age. The incidence of chronic ITP ranges from 3.2 to 6.6 cases per 100,000 per year.⁴¹ Chronic ITP usually begins insidiously, with platelet counts that are variably decreased and sometimes normal for periods of time. Presenting symptoms are those of mucocutaneous bleeding, with menorrhagia, recurrent epistaxis, and easy bruising (ecchymoses) being most common.

Platelet destruction in chronic ITP is the result of an immunologic process. The offending antibodies attach to platelets, and as a result, the antibody-labeled platelets are removed from the circulation by reticuloendothelial cells, primarily in the spleen. Autoantibodies that recognize platelet surface glycoproteins such as glycoprotein IIb (GP IIb) and GP IIIa ($\alpha\text{IIb}/\beta_3$), GP Ia/IIa, and others can be demonstrated in 50% to 60% of ITP patients.^{42,43} Because megakaryocytes also express GP IIb/IIIa and GP Ib/IX on their membranes, these cells are obvious targets of the antibodies. Platelet turnover studies have shown impaired platelet production in ITP. Overall, the life span of the platelet is shortened from the normal 7 to 10 days to a few hours, and the rapidity with which platelets are removed from the circulation correlates with the degree of thrombocytopenia. If plasma from a patient with ITP is infused into the circulation of a normal recipient, the recipient develops thrombocytopenia. The thrombocytopenia-producing factor in the plasma of the ITP patient is an immunoglobulin G (IgG) antibody that can be removed from serum by adsorption with normal human platelets. In addition, cytotoxic T cell-mediated lysis of platelets has been shown in vitro using $\text{CD}3^+\text{CD}8^+$ lymphocytes from patients with active chronic ITP, although the in vivo significance of this mechanism is not known.⁴⁴

The only abnormalities in the peripheral blood of patients with ITP are related to platelets. In most cases, platelets number between 30,000/ μL and 80,000/ μL . Patients with ITP undergo periods of remission and exacerbation, however, and their platelet counts may range from near normal to fewer than 20,000/ μL during these periods (Figure 40-3). Morphologically, platelets appear normal, although larger in diameter than usual. This is reflected in an increased mean platelet volume as measured by electronic cell counters. The marrow typically is characterized by megakaryocytic hyperplasia. Megakaryocytes are increased in size, and young forms with a single nucleus,

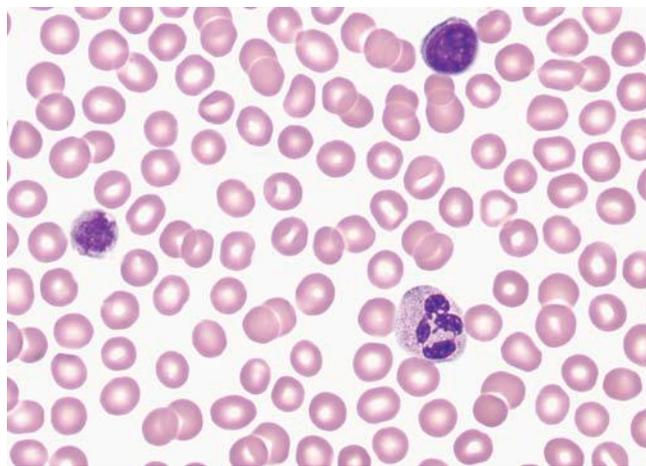


Figure 40-3 Typical peripheral blood cell morphology in immune thrombocytopenic purpura. Note scarce platelets and increased platelet size but normal red blood cell and leukocyte morphology (peripheral blood, $\times 500$).

smooth contour, and diminished cytoplasm are commonly seen. In the absence of bleeding, infection, or other underlying disorder, erythrocyte and leukocyte precursors are normal in number and morphology. Coagulation tests showing abnormal results include tests dependent on platelet function. Although platelet-associated IgG levels are increased in most patients,^{10,21,45} it has not been shown conclusively that any method of testing for platelet antibodies is sensitive or specific for ITP.

The initial treatment of chronic ITP depends on the urgency for increasing the platelet count. In ITP patients with platelet counts greater than 30,000/ μL who receive no treatment, the expected mortality rate is equal to that of the general population. Unless there are additional risk factors, ITP patients with platelet counts greater than 30,000/ μL should not be treated. If additional risk factors are present, such as old age, coagulation defects, recent surgery, trauma, or uncontrolled hypertension, the platelet count should be kept at 50,000/ μL or higher, depending on the clinical situation. In patients in whom the need is considered urgent, IVIG remains the treatment of choice. For most patients, however, the initial treatment of chronic ITP consists principally of prednisone. About 70% to 90% of patients respond to this therapy, with an increase in platelet count and a decrease in hemorrhagic episodes. Although reported response rates vary widely, about 50% of patients have a long-term beneficial effect from corticosteroid treatment.⁴⁶ If the response to corticosteroids is inadequate or no response is seen, steroid therapy can be supplemented with IVIG or, in some cases, anti-D immunoglobulin.⁴⁷ For patients in whom prednisone is ineffective, intravenous rituximab should be tried. Responses to rituximab are usually seen within 3 to 4 weeks. In some patients splenectomy may become necessary. Splenectomy eliminates the primary site of platelet removal and destruction, but it also removes an organ containing autoantibody-producing lymphocytes. Splenectomy is an effective treatment for adult chronic ITP, with 88% of patients

showing improvement and 66% having a complete and lasting response.⁴⁸ Vaccination with pneumococcal, meningococcal, and *Haemophilus influenzae* vaccines should be performed at least 2 weeks prior to surgery. The use of laparoscopic surgery speeds recovery and shortens hospitalization and is generally preferred to open splenectomy. In the most severe refractory cases, immunosuppressive (chemotherapeutic) agents such as azathioprine given alone or with steroids may be necessary. In such patients, platelet transfusions may be of transient benefit in treating severe hemorrhagic episodes but should not be given routinely.⁴⁵ IVIG given alone or just before platelet transfusion also may be beneficial.^{37,45}

Chronic ITP occurring in association with HIV infection, with hemophilia, or with pregnancy presents special problems in diagnosis and therapy. Unexplained thrombocytopenia in otherwise healthy members of high-risk populations may be an early manifestation of acquired immune deficiency syndrome (AIDS).^{36,45}

Differentiation of Acute Versus Chronic Immune Thrombocytopenic Purpura. The differences between acute and chronic ITP are summarized in Table 40-3. Acute ITP occurs most frequently in children 2 to 9 years of age and in young adults, whereas chronic ITP occurs in patients of all ages, although most frequently in adults aged 20 to 50 years, and more commonly in women. Of patients with acute ITP, 60% to 80% have a history of infection, usually viral (rubella, rubeola, chickenpox, and nonspecific respiratory tract infection), occurring 2 to 21 days before ITP onset. Acute ITP also may occur after immunization with live vaccine for measles, chickenpox, mumps, and smallpox.

TABLE 40-3 Clinical Picture of Acute and Chronic Immune Thrombocytopenic Purpura

Characteristic	Acute	Chronic
Age at onset	2–6 yr	20–50 yr
Sex predilection	None	Female over male, 3:1
Prior infection	Common	Unusual
Onset of bleeding	Sudden	Gradual
Platelet count	<20,000/ μ L	30,000–80,000/ μ L
Duration	2–6 wk	Months to years
Spontaneous remission	90% of patients	Uncommon
Seasonal pattern	Higher incidence in winter and spring	None
Therapy		
Steroids	70% response rate	30% response rate
Splenectomy	Rarely required	<45 yr, 90% response rate >45 yr, 40% response rate

From Triplett DA, editor: *Platelet function: laboratory evaluation and clinical application*, Chicago, 1978, American Society of Clinical Pathologists; Quick AJ: *Hemorrhagic diseases and thrombosis*, ed 2, Philadelphia, 1966, Lea & Febiger; and Bussel J, Cines D: Immune thrombocytopenia, neonatal alloimmune thrombocytopenia, and post-transfusion purpura. In Hoffman R, Benz EJ Jr, Shattil SJ, et al, editors: *Hematology: basic principles and practice*, ed 3, New York, 2000, Churchill Livingstone, pp 2096-2114.

Acute ITP usually is self-limited, and spontaneous remissions occur in 80% to 90% of patients, although the duration of the illness may range from days to months. In chronic ITP, there is typically a fluctuating clinical course, with episodes of bleeding that last a few days or weeks, but spontaneous remissions are uncommon and usually incomplete.⁴⁵

Symptoms of acute ITP vary, but petechial hemorrhages, purpura, and often bleeding from the gums and gastrointestinal or urinary tract typically begin suddenly, sometimes over a few hours. Hemorrhagic bullae in the oral mucosa are often prominent in patients with severe thrombocytopenia of acute onset. Usually the severity of bleeding is correlated with the degree of thrombocytopenia.⁴⁵ In contrast, presenting symptoms of chronic ITP begin with a few scattered petechiae or other minor bleeding manifestations. Occasionally, a bruising tendency, menorrhagia, or recurrent epistaxis is present for months or years before diagnosis. Platelet counts range from 5000/ μ L to 75,000/ μ L and are generally higher than those in acute ITP. Giant platelets are commonly seen. Platelet-associated immunoglobulin levels are elevated in most patients, but the test lacks sensitivity and specificity.⁴⁵

Treatment also varies for acute and chronic ITP. In chronic ITP, initial therapy often consists of glucocorticoids (corticosteroids), which interfere with splenic and hepatic macrophages to increase platelet survival time. If the ITP does not respond to corticosteroids or the patient cannot tolerate them because of the resultant immunosuppression and toxicity, splenectomy may be necessary. In acute ITP, treatment for all but the most severely thrombocytopenic and hemorrhagic patients is contraindicated. When treatment is necessary, a good response to IVIG or corticosteroids or both usually can be obtained, and splenectomy is rarely required.^{37,38}

Immunologic Drug-Induced Thrombocytopenia. As can be seen from Box 40-2, many drugs can induce acute thrombocytopenia. Drug-induced immune-mediated thrombocytopenia can be divided into several types based on the interaction of the antibody with the drug and platelets. Mechanisms of drug-antibody binding are shown in Figure 40-4.

Drug-dependent antibodies. One mechanism of drug-dependent antibodies is typified by quinidine- and quinine-induced thrombocytopenia and has been recognized for more than 100 years. The antibody induced by drugs of this type interacts with platelets only in the presence of the drug. Drug-dependent antibodies typically occur after 1 to 2 weeks of exposure to a new drug. Many drugs can induce such antibodies, but quinine, quinidine, and sulfonamide derivatives do so more often than other drugs. When antibody production has begun, the platelet count falls rapidly and often may be fewer than 10,000/ μ L. Patients may have abrupt onset of bleeding symptoms. If this type of drug-induced thrombocytopenia develops in a pregnant woman, both she and her fetus may be affected. Quinine previously was used to facilitate labor but is no longer used for this purpose.

The initial studies of quinidine-induced thrombocytopenia suggested that the drug first combines with the antibody and that the antigen-antibody (immune) complex then attaches to

BOX 40-2 Common Drugs Causing Immune Thrombocytopenia

Analgesics

Salicylates
Acetaminophen
Phenylbutazone

Antibiotics

Cephalothin
Penicillin
Streptomycin
Aminosalicylic acid
Rifampin
Novobiocin
Various sulfa drugs (chlorthalidone, furosemide)

Alkaloids

Quinidine
Quinine

Sedatives, Anticonvulsants

Methoin
Troxidone
Chlorpromazine
Diphenylhydantoin
Meprobamate
Phenobarbital
Carbamazepine

Oral Hypoglycemics

Chlorpropamide
Tolbutamide

Heavy Metals

Gold
Mercury
Bismuth
Organic arsenicals

Miscellaneous

Chloroquine
Chlorothiazide
Insecticides

From Triplett DA, editor: *Platelet function: laboratory evaluation and clinical application*, Chicago, 1978, American Society of Clinical Pathologists; and Quick AJ: *Hemorrhagic diseases and thrombosis*, ed 2, Philadelphia, 1966, Lea & Febiger.

the platelet in an essentially nonspecific manner (the “innocent bystander” hypothesis). It now seems clear, however, that the antibodies responsible for drug-induced thrombocytopenia bind to the platelets by their Fab regions, rather than by attaching nonspecifically as immune complexes. The innocent bystander/immune complex explanation for this type of drug-induced thrombocytopenia should be abandoned. The Fab portion of the antibody binds to a platelet membrane constituent,

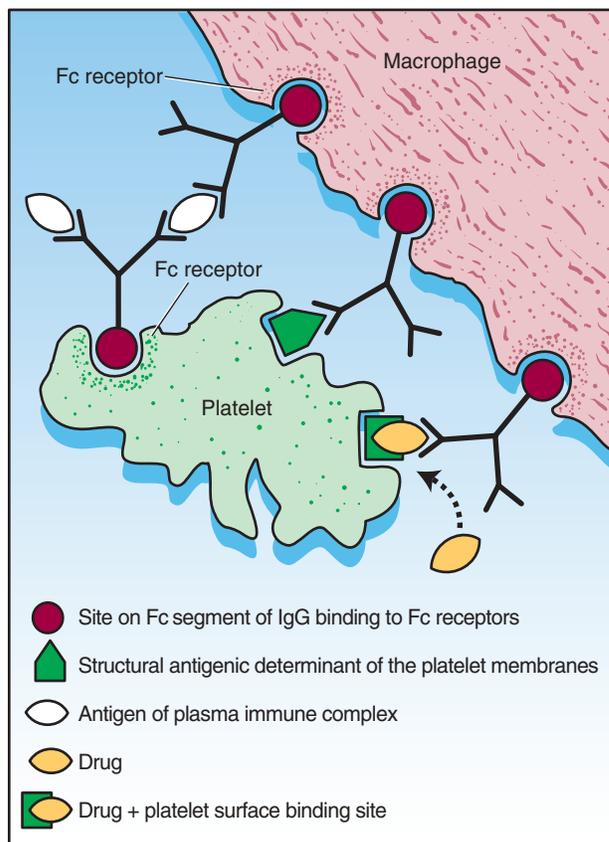


Figure 40-4 Immunoglobulin binds a platelet membrane antigen or antigen and drug combination. Macrophage Fc receptors bind the Fc portion of the immunoglobulin. This may result in platelet removal and thrombocytopenia. *IgG*, immunoglobulin G. (From Rapaport SI: *Introduction to hematology*, ed 2, Philadelphia, 1987, JB Lippincott, p. 489.)

usually the GP Ib/IX/V complex or the GP IIb/IIIa complex, only in the presence of drug.^{49,50} The mechanism by which the drug promotes binding of a drug-dependent antibody to a specific target on the platelet membrane without covalently linking to the target or the antibody remains to be determined, however. Because the Fc portion of the immunoglobulin is not involved in binding to platelets, it is still available to the Fc receptors on phagocytic cells. This situation may contribute to the rapid onset and relatively severe nature of the thrombocytopenia. Most drug-induced platelet antibodies are of the IgG class, but in rare instances, IgM antibodies are involved.⁴⁵

A similar pattern is seen with the antiplatelet/antithrombotic agents abciximab, tirofiban, and eptifibatid, although with these drugs thrombocytopenia tends to occur within several hours of exposure. Such immediate reactions are due to naturally occurring antibodies to the murine structural elements of abciximab (a mouse/human monoclonal antibody fragment) or to structural changes induced by binding of eptifibatid or tirofiban to platelet GP IIb/IIIa.

Hapten-induced antibodies. A second mechanism of drug-induced thrombocytopenia is induction of hapten-dependent antibodies. Some drug molecules are too small by themselves to trigger an immune response, but they may act as

a hapten and combine with a larger carrier molecule (usually a plasma protein or protein constituent of the platelet membrane) to form a complex that can act as a complete antigen.⁴⁵ Penicillin and penicillin derivatives are the primary offending agents causing drug-induced thrombocytopenia by this mechanism. Drug-induced thrombocytopenia of this type is often severe. The initial platelet count may be fewer than 10,000/ μL and sometimes fewer than 1000/ μL . The number of bone marrow megakaryocytes is usually normal to elevated.⁴⁵ Bleeding is often severe and rapid in onset, and hemorrhagic bullae in the mouth may be prominent.

Drug-induced autoantibodies. Drug-induced autoantibodies represent a third mechanism of drug-induced thrombocytopenia. In this case, the drugs stimulate the formation of an autoantibody that binds to a specific platelet membrane glycoprotein with no requirement for the presence of free drug. Gold salts and procainamide are two examples of such drugs. Levodopa also may cause thrombocytopenia in the same way. The precise mechanism by which these drugs induce autoantibodies against platelets is not known with certainty.

Treatment for any drug-induced thrombocytopenia is first to identify the offending drug, immediately discontinue its use, and substitute another suitable therapeutic agent. This is often difficult to accomplish. Many patients are taking multiple drugs, and it is not always easy to determine which of the drugs is at fault for causing thrombocytopenia. Under these conditions, identifying the causative agent may be a trial-and-error procedure in which the most likely drugs are eliminated one at a time. In addition, even if the patient is taking only one agent, there may not be a suitable replacement, or a prolonged period may be required for the alternative drug to become effective. Drugs usually are cleared from the circulation rapidly, but dissociation of drug-antibody complexes may require longer periods, perhaps 1 to 2 weeks.¹⁰ In some cases, such as those caused by gold salts, thrombocytopenia may persist for months. Platelet transfusions may be necessary for patients with life-threatening bleeds. Although it is true that the transfused platelets are destroyed rapidly, they may function to halt bleeding effectively before they are destroyed. In addition, high-dose

IVIg may be used and is generally an effective treatment for most drug-induced immune thrombocytopenias. Laboratory testing to identify the specific drug involved is usually beyond the capabilities of most laboratories. This type of testing is performed by many reference laboratories, however.

Immune complex-induced thrombocytopenia. *Heparin-induced thrombocytopenia* (HIT) is a good example of another type of drug-induced thrombocytopenia. Heparin binds to platelet factor 4 (PF4), a heparin-neutralizing protein made and released by platelets (Figure 40-5). Binding of heparin by plasma PF4 or platelet membrane-expressed PF4 causes a conformational change in PF4, resulting in the exposure of neoepitopes. Exposure of these neoepitopes (“new antigens”) stimulates the immune system of some individuals, which leads to the production of an antibody to one of the neoepitopes. In HIT, heparin and PF4 form a complex on the platelet surface or circulating free complexes to which the antibody binds. The Fab portion of the immunoglobulin molecule binds to an exposed neoepitope in the PF4 molecule; this leaves the Fc portion of the IgG free to bind with the platelet Fc γ IIa receptor, which causes platelet activation.^{51,52} Because the Fc portions of the IgG molecules bind to platelet Fc γ IIa receptor, they are not available to the Fc receptors of the cells of the reticuloendothelial system. This may explain the less severe decline in platelet count in this thrombocytopenia. That does not mean, however, that the consequences are less serious. The opposite may be true. Because platelets are activated by occupancy of their Fc γ IIa receptor, in vivo platelet aggregation with thrombosis is possible. HIT sometimes is referred to as *heparin-induced thrombocytopenia and thrombosis* (Chapter 39). Heparin binding to PF4 is required to expose the neoepitope to which the antibody binds. The treatment for HIT is to discontinue heparin administration and replace it with another suitable anticoagulant. Low-molecular-weight heparin should not be used as a heparin replacement for this purpose, because the antibody cross-reacts with low-molecular-weight heparin and PF4 to result in platelet activation and aggregation.⁵³

Heparin-induced thrombocytopenia. HIT is a relatively common side effect of unfractionated heparin administration,

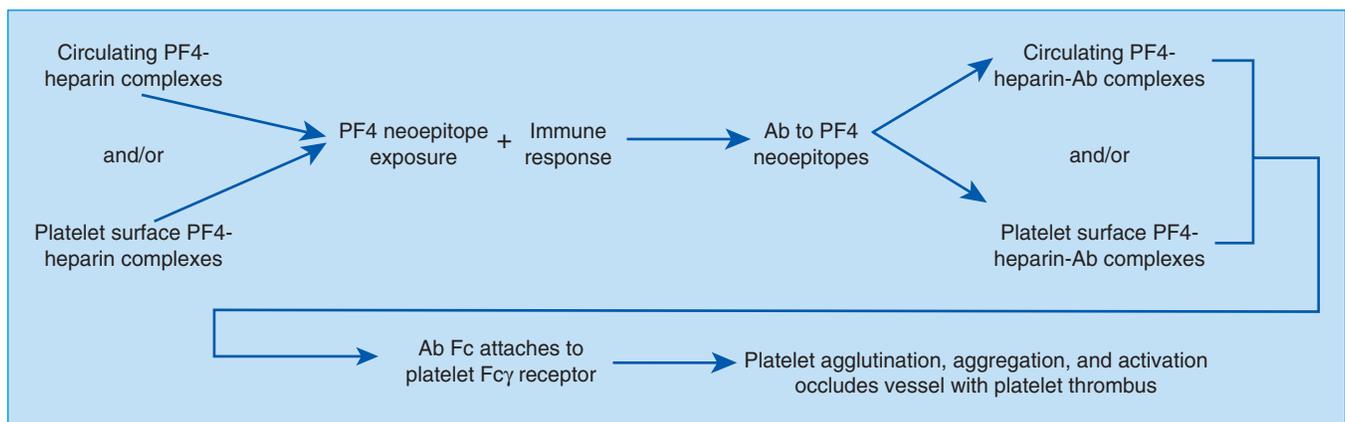


Figure 40-5 Heparin-induced thrombocytopenia with thrombosis. An antibody (Ab) binds the heparin–platelet factor 4 (PF4) complex in plasma or on the platelet surface. The Fc portion binds platelet Fc receptors and activates the platelet. The activated platelets aggregate to form platelet thrombi in the arterial circulation. Thrombi can also occur in the venous system.

with about 1% to 5% of patients developing this complication. Despite the thrombocytopenia, patients with HIT usually are not at significant risk of bleeding, because the platelet count typically does not fall below 40,000/ μ L. Ten percent to 30% of patients with HIT develop thrombotic complications, however. In patients who develop HIT, heparin therapy should be stopped as soon as the diagnosis is made, because continued heparin therapy can lead to significant morbidity and mortality, including gangrene of the extremities, amputation, and death. After discontinuation of heparin, the platelet count begins to increase and should return to normal within a few days.⁵⁴

Because the immune system is involved in the development of HIT, the clinical signs of HIT typically are not seen until 7 to 14 days after the initiation of heparin therapy (the time necessary to mount an immune response on first exposure to an antigen). If the patient has been exposed to heparin previously, however, symptoms of HIT may be seen in 1 to 3 days. Because the platelet count may fall sharply in 1 day, it is recommended that platelet counts be measured daily in patients receiving unfractionated heparin therapy (Table 40-4).

One other sign of impending HIT in some patients is the development of *heparin resistance*. This is the clinical situation in which a patient who had experienced adequate anticoagulation

at a certain heparin dosage suddenly requires increasing amounts of heparin to maintain the same level of anticoagulation. This situation can result from *in vivo* activation of platelets and release of PF4 and β -thromboglobulin from platelet α -granules. Both of these substances neutralize heparin, which leads to a normalization of results on the partial thromboplastin time test that is used to monitor heparin therapy. Heparin resistance often is seen before the development of thrombocytopenia.⁵⁵

A common benign form of HIT that occurs on heparin administration is type I (non-immune mediated) HIT. It is important to distinguish benign type I from type II HIT. *Type I HIT* is associated with a rapid decrease in the platelet count after administration of heparin, but the thrombocytopenia is mild (the platelet count rarely decreases to fewer than 100,000/ μ L) and transient, and the platelet count returns rapidly to the preheparin level even if heparin therapy is continued. Careful attention should be paid to the platelet count and other signs of HIT so that this form of thrombocytopenia is not confused with the clinically significant *type II HIT*. Although the mechanism of type I HIT has not been completely described, it may be related to the well-documented proaggregatory effects of heparin.^{53,56} Because activated or aggregated platelets are cleared from the circulation, these effects may explain the mild decrease in the platelet count that occurs during the first few days of heparin administration. This has not been clearly documented, however.

The binding of heparin and related compounds depends on polysaccharide chain length, composition, and degree of sulfation. Short-chain heparin polysaccharides (low-molecular-weight heparin) have lower affinity to PF4 and are less prone to cause type II HIT. Pentasaccharide and its synthetic derivatives (e.g., fondaparinux) do not seem to bind PF4 and are unlikely to cause type II HIT.

The detection of clinically significant HIT occurs by laboratory testing using immunoassays and platelet function tests (Table 40-4 and Chapter 42). Laboratory testing, however, is problematic because all tests lack sensitivity. Three methods are commonly used, but all depend on the presence of free heparin-induced antiplatelet antibodies in the patient's serum or plasma in sufficient quantity to cause a positive test result. HIT can be detected by a *platelet aggregation technique*.⁵⁷ In this method, serum from the patient is added to platelet-rich plasma from normal donors, heparin is added to the mixture, and platelet aggregation is typically monitored for 20 minutes. The specificity of the method is excellent (near 100%), but the sensitivity is quite low (about 50%). The sensitivity of the test can be improved, but this requires the use of several heparin concentrations and platelet-rich plasma from two or more blood donors, preferably of the same ABO blood type as the patient. In addition, the individuals donating blood for platelet-rich plasma must not have taken aspirin for 10 to 14 days before platelet donation because platelets from donors who have ingested aspirin produce a false-negative result for HIT.⁵³ In the author's experience, patients who develop HIT while receiving aspirin therapy rarely develop thrombotic complications, and the drop in their platelet counts is less precipitous, gradually decreasing over the course of several days

TABLE 40-4 Laboratory Tests for Heparin-Induced Thrombocytopenia

Laboratory Test	Comment
Platelet count	A >30% decrease from baseline may signal HIT, even if still within the reference interval.
Antigen Tests	
ELISAs:	Use with a clinical scoring system (Table 39-17). Can be used as a first lab test to screen for the presence of HIT antibodies; due to high frequency of false positive results, functional tests should be performed to confirm a positive antigen test. False negative results can also occur.
GTI PVS:PF4*	
Stago H:PF4*	
Hyphen BioMed	
Rapid tests (point-of-care):	
Akers PIFA*	Important to perform functional testing that detects platelet activation by HIT antibodies to confirm a diagnosis of HIT; sensitivities and specificities differ among tests with the SRA (washed platelet assay) being the most sensitive. Require skill and experience of the operator to obtain quality results.
DiaMed Pa-GIA	
Coagulation instrument based tests:	
Milenia Biotec LFI-HIT	Require skill and experience of the operator to obtain quality results.
HemosIL HIT-Ab	
Functional Tests	
Platelet Aggregation HIPA	Important to perform functional testing that detects platelet activation by HIT antibodies to confirm a diagnosis of HIT; sensitivities and specificities differ among tests with the SRA (washed platelet assay) being the most sensitive. Require skill and experience of the operator to obtain quality results.
Lumi-Aggregation HIPA	
Serotonin Release Assay	

HIPA, Heparin-induced platelet aggregation.

*FDA-cleared.

(unpublished observations). Given the efficacy of aspirin therapy in primary and secondary prevention of myocardial infarction and thrombotic stroke, it is increasingly difficult to find a sufficient pool of suitable (and willing) blood donors. Although the platelet aggregation procedure is time consuming, reasonable sensitivity can be obtained with sufficient attention to the details of the technique.²⁷

Dense granules of platelets contain serotonin, and platelets have an active mechanism for rapid uptake and storage of serotonin in dense granules. This property of platelets is used in another test for HIT, the *serotonin release assay* (SRA), in which washed normal platelets from a donor are incubated with radioactive serotonin (Figure 40-6).⁵⁸ Radioactive serotonin is taken up rapidly and stored in the dense granules of the donor platelets, which are washed and resuspended. In the presence of heparin-dependent antiplatelet antibody and heparin, the donor platelets become activated and release the contents of their dense granules when the concentration of heparin in the test suspension is near the therapeutic range. The reappearance of radioactive serotonin in the plasma indicates the presence of a

heparin-dependent antiplatelet antibody (i.e., HIT). Under these same conditions, supratherapeutic concentrations of heparin do not activate platelets, however, and if platelets release the contents of dense granules at both therapeutic and supratherapeutic concentrations of heparin, the test result is not positive for HIT. A similar phenomenon is observed in the test that uses platelet aggregometry.^{53,59} The SRA is considered the gold standard for detection of HIT. Its major drawback is the requirement for radiolabeled serotonin. Most clinical laboratories no longer use isotopic techniques. As a result, this test is performed only in a few specialized centers. In addition, it has some of the same methodologic drawbacks as the platelet aggregation technique, in particular the need for platelets from drug-free donors. Nonetheless, when properly performed, this technique has similar specificity and superior sensitivity compared with the platelet aggregation method.

More recently, *enzyme-linked immunosorbent assays* (ELISAs) have been developed based on the knowledge that the antigenic target of the heparin-dependent antiplatelet antibody is a PF4-heparin complex (Figure 40-7). In this assay, PF4 and

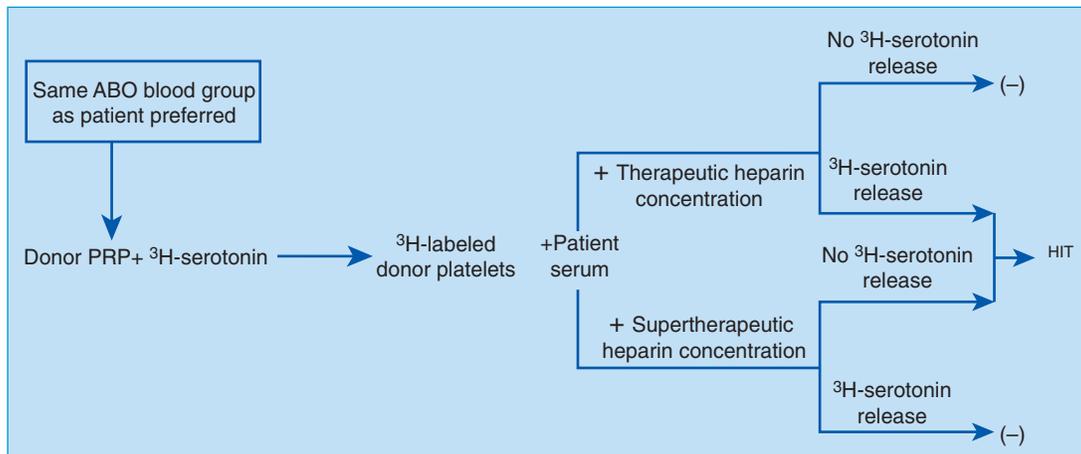


Figure 40-6 The serotonin release assay (SRA) for heparin-induced thrombocytopenia (HIT). Donor platelets in platelet-rich plasma (PRP) are labeled with tritiated (^3H) serotonin, washed, and suspended in a buffer to which patient plasma is added. Heparin in therapeutic and saturating doses is added to two aliquots. Release of radioactive serotonin in the therapeutic aliquot in combination with no release in the supratherapeutic system indicates HIT.

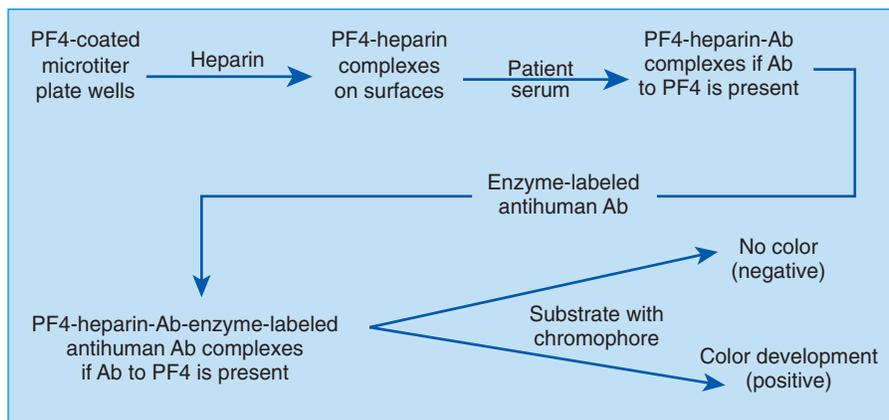


Figure 40-7 Enzyme immunoassay for heparin-induced thrombocytopenia (HIT). The solid-phase target antigen is a complex of platelet factor 4 (PF4) and heparin or a heparin surrogate. Anti-heparin-PF4 antibody in patient serum binds the antigen and is bound by enzyme-labeled anti-human antibody (Ab), a “sandwich” assay. The enzyme catalyzes the release of a chromophore from its substrate.

heparin (or a related compound) are coated to the surfaces of microplate wells. The serum or plasma from the patient suspected to have HIT is added to wells of the microtiter plate. If the antibody is present, it adheres to the PF4-heparin (or heparin-like compound) complex. The plate wells are washed, and enzyme-labeled monoclonal antibodies against human IgG and IgM are added. After an appropriate incubation period, the plate is washed, and a chromogenic substrate for the enzyme is added. Color development in the assay well indicates the presence of the heparin-dependent antiplatelet antibody in the patient specimen.⁶⁰ This assay has greater sensitivity than the platelet aggregation method and similar sensitivity to the SRA, but it has lower specificity than the serotonin release assay or the platelet aggregation method. Unlike the functional HIT assays (SRA and platelet aggregation), the ELISA can detect anti-PF4-heparin antibodies that are not pathologic; that is, the test result is positive, but the patient does not have clinical HIT. Some ELISA assays can detect both IgG and IgM antibodies to heparin-PF4 complexes. More recently, ELISA kits that detect only IgG antibodies have been developed and have a better correlation with the SRA. The ELISA method is considerably less labor intensive, does not require blood from healthy drug-free donors, and can be performed in most laboratories.

For patients who develop type II HIT, it is essential that heparin therapy be withdrawn immediately. It is not prudent, however, to discontinue administration of an anticoagulant/antithrombotic without substituting a suitable alternative. It is clear from the literature that under these circumstances, withdrawal of heparin treatment without replacement anticoagulant therapy results in an unacceptably high rate of thrombotic events. In the recent past, good alternatives for heparin were not available. Today, several alternative agents are suitable substitutes (although considerably more expensive), including *direct thrombin inhibitors* such as the intravenous use of argatroban and bivalirudin (Angiomax) (Chapter 43). Fondaparinux (Arixtra) is a synthetic heparin pentasaccharide, identical in chemical structure to the antithrombin binding sequence in heparin and heparin-derived agents such as low-molecular-weight heparins. Fondaparinux is given subcutaneously, and while its use in patients with HIT is "off-label" (not FDA approved), it is gaining favor in the clinical community as a second-line agent for the management of suspected HIT to avoid progression into acute HIT.

Neonatal Alloimmune Thrombocytopenia. Neonatal alloimmune thrombocytopenia (NAIT) develops when the mother lacks a platelet-specific antigen (usually human platelet antigen 1a, or HPA-1a [P1^{A1}]) that the fetus has inherited from the father. HPA-1a is the most often involved (80% of cases), and HPA-5b accounts for another 10% to 15% of cases. Fetal platelet antigens may pass from the fetal to the maternal circulation as early as the fourteenth week of gestation.⁶¹ If the mother is exposed to a fetal antigen she lacks, she may make antibodies to that fetal antigen. These antibodies cross the placenta, attach to the antigen-bearing fetal platelets, and result in thrombocytopenia in the fetus. In this regard, the pathophysiology of NAIT is the same as that of hemolytic disease of the newborn.

The most frequent cause of NAIT in whites is the HPA-1a antigen expressed on GP IIIa of the surface membrane GP IIb/IIIa complex, followed by HPA-5b (Br^a). The antigen HPA-3a (Bak^a) is present on GP IIb and is an important cause of neonatal thrombocytopenia in Asians. Platelet antigen HPA-4 (Penn and Yuk) accounts for the disorder in a few affected neonates.

Clinically significant thrombocytopenia develops in an estimated 1 in 1000 to 2000 newborns.⁶² With the first pregnancy, about 50% of neonates born to mothers lacking a specific platelet antigen are affected, whereas with subsequent pregnancies the risk is 75% to 97%.⁶² The incidence of intracranial hemorrhage or death or both in affected offspring is about 25%, and about half of the intracranial hemorrhages occur in utero in the second trimester.

Affected infants may appear normal at birth but soon manifest scattered petechiae and purpuric hemorrhages. In many infants with NAIT, serious hemorrhage does not develop, however, and the infants recover over a 1- to 2-week period as the level of passively transferred antibody decreases.^{10,38} In symptomatic cases, platelet levels are usually below 30,000/ μ L and may diminish even further in the first few hours after birth.

The diagnosis of NAIT is one of exclusion of other causes of neonatal thrombocytopenia, including maternal ITP and maternal ingestion of drugs known to be associated with drug-induced thrombocytopenia. The presence of thrombocytopenia in a neonate with a HPA-1a-negative mother or a history of the disorder in a sibling is strong presumptive evidence in favor of the diagnosis. Confirmation should include platelet typing of both parents and testing for evidence of a maternal antibody directed at paternal platelets.³⁷

In situations in which suspicion of NAIT is high or there is a history of NAIT in a first pregnancy, it may be necessary to test or treat the fetus to prevent intracranial hemorrhage in utero. Fetal genotypes now can be determined at 10 to 18 weeks of gestation using polymerase chain reaction methods on cells obtained by chorionic villus sampling or amniocentesis.⁶³ Periumbilical sampling to determine the fetal platelet count can be performed at about 20 weeks of gestation. When the fetus is thrombocytopenic, weekly maternal infusions of IVIG have been shown to be effective in increasing the fetal platelet count in most cases.⁶⁴ In cases in which the fetal platelet count does not increase with IVIG therapy, washed maternal platelets have been infused into the fetus with good results.⁶⁵ Treatment of the mother with high-dose corticosteroids (to decrease maternal antibody production) is not recommended because of potential fetal toxicity. In situations in which the diagnosis of NAIT is known or highly suspected, delivery should be by cesarean section to avoid fetal trauma associated with vaginal delivery. After delivery, the affected infant may be treated with transfusion of the appropriate antigen-negative platelets (usually maternal). In addition, IVIG can be used alone or in combination with platelet transfusion. IVIG should not be used as the sole treatment in a bleeding infant, because response to this therapy usually takes 1 to 3 days.⁶²

Neonatal Autoimmune Thrombocytopenia. The diagnosis of ITP or systemic lupus erythematosus in the mother is a prerequisite for the diagnosis of neonatal autoimmune thrombocytopenia. Neonatal autoimmune thrombocytopenia is due to passive transplacental transfer of antibodies from a mother with ITP or, occasionally, systemic lupus erythematosus. The neonate does not have an ongoing autoimmune process per se, but rather is an incidental target of the mother's autoimmune process. During pregnancy, relapse is relatively common for women with ITP in complete or partial remission; this has been attributed to the facilitation of reticuloendothelial phagocytosis by the high estrogen levels in pregnant women. Women commonly develop chronic ITP during pregnancy. ITP in the mother tends to remit after delivery. Corticosteroids are the primary treatment for pregnant women with ITP, and at the dosages used, there is a relatively low incidence of adverse fetal side effects.⁶⁶ Neonatal autoimmune thrombocytopenia develops in only about 10% of the infants of pregnant women with autoimmune thrombocytopenia, and intracranial hemorrhage occurs in 1% or less. It is no longer recommended that high-risk infants be delivered by cesarean section to avoid the trauma of vaginal delivery and accompanying risk of hemorrhage in the infant, regardless of maternal platelet count.⁶⁷

Affected newborns may have normal to decreased platelet numbers at birth and have a progressive decrease in the platelet count for about 1 week before the platelet count begins to increase. It has been speculated that the falling platelet count is associated with maturation of the infant's reticuloendothelial system and accelerated removal of antibody-labeled platelets by cells of the reticuloendothelial system. Neonatal thrombocytopenia typically persists for about 1 to 2 weeks but sometimes lasts for several months. It usually does not require treatment. Severely thrombocytopenic infants generally respond quickly to IVIG treatment. If an infant develops hemorrhagic symptoms, platelet transfusion, IVIG treatment, or corticosteroid therapy should be started immediately.³⁷

Posttransfusion Purpura. Posttransfusion purpura (PTP) is a relatively rare disorder that typically develops about 1 week after transfusion of platelet-containing blood products, including fresh or frozen plasma, whole blood, and packed or washed RBCs. PTP is manifested by the rapid onset of severe thrombocytopenia and moderate to severe hemorrhage that may be life-threatening. The recipient's plasma is found to contain alloantibodies to antigens on the platelets or platelet membranes of the transfused blood product, directed against an antigen the recipient does not have. In more than 90% of cases, the antibody is directed against the HPA-1a antigen; in most of the remaining cases, the antibodies are directed against PI^{A2} or other epitopes on GP IIb/IIIa.³⁷ Involvement of other alloantigens, such as HPA-3a (Bak), HPA-4 (Penn), and HPA-5b (Br), has been reported. The mechanism by which the recipient's own platelets are destroyed is unknown. Most patients with this type of thrombocytopenia are multiparous middle-aged women. Almost all the other patients have a history of blood transfusion. PTP seems to be exceedingly rare in men who have

never been transfused and in women who have never been pregnant or never been transfused.⁶⁸ PTP seems to require prior exposure to foreign platelet antigens and behaves in many respects like an anamnestic immune response.

No clinical trials have been conducted on the treatment of PTP, primarily because of the small number of cases. If PTP is untreated or treatment is ineffective, mortality rates may approach 10%.⁶⁹ In addition, untreated or unresponsive patients have a protracted clinical course, with thrombocytopenia typically lasting 3 weeks but in some cases up to 4 months. Plasmapheresis and exchange transfusion have been used with some success in the past, but the treatment of choice is now IVIG. Many patients with PTP respond to a 2-day course of IVIG, generally within the first 2 to 3 days, although a second course occasionally is necessary.⁶⁹ IVIG also is much easier to administer, and the response rates are higher than for plasmapheresis or exchange transfusion. Corticosteroid therapy is not particularly efficacious when used alone but may be beneficial in combination with other, more effective treatments.³⁷

Secondary Thrombocytopenia, Presumed to Be Immune Mediated.

Severe thrombocytopenia has been observed in patients receiving biologic response modifiers such as interferons, colony-stimulating factors, and interleukin-2.⁷⁰⁻⁷² The thrombocytopenia associated with use of these substances is reversible and, at least for interferon, may be immune mediated, because increased levels of platelet-associated IgG have been measured. Immune thrombocytopenia develops in about 5% to 10% of patients with chronic lymphocytic leukemia and in a smaller percentage of patients with other lymphoproliferative disorders.^{73,74} Thrombocytopenia also is noted in 14% to 26% of patients with systemic lupus erythematosus.⁷⁵ The clinical picture is similar to that of ITP: the bone marrow has a larger than normal number of megakaryocytes, and increased levels of platelet-associated IgG frequently are found.³⁶ Parasitic infections also are known to cause thrombocytopenia. Malaria is the most studied in this group and is regularly accompanied by thrombocytopenia, the onset of which corresponds to the first appearance of antimalarial antibodies, a decrease in serum complement, and control of parasitemia. There is evidence for the adsorption of microbial antigens to the platelet surface and subsequent antibody binding via the Fab terminus.⁷⁶ Immune destruction of platelets seems to be the most likely mechanism for the thrombocytopenia.

Nonimmune Mechanisms of Platelet Destruction

Nonimmune platelet destruction may result from exposure of platelets to nonendothelial surfaces, from activation of the coagulation process, or from platelet consumption by endovascular injury without measurable depletion of coagulation factors.³⁶

Thrombocytopenia in Pregnancy and Preeclampsia

Incidental Thrombocytopenia of Pregnancy. Incidental thrombocytopenia of pregnancy also is known as pregnancy-associated thrombocytopenia and gestational thrombocytopenia. This disorder is the most common cause of thrombocytopenia in pregnancy. Random platelet counts in pregnant and postpartum

women are slightly higher than normal, but about 5% of pregnant women develop a mild thrombocytopenia (100,000 to 150,000/ μ L), with 98% of such women having platelet counts greater than 70,000/ μ L. These women are healthy and have no prior history of thrombocytopenia. They do not seem to be at increased risk for bleeding or for delivery of infants with neonatal thrombocytopenia. The cause of this type of thrombocytopenia is unknown. Maternal platelet counts return to normal within several weeks of delivery. These women commonly experience recurrence in subsequent pregnancies.

Preeclampsia and other hypertensive disorders of pregnancy. Approximately 20% of cases of thrombocytopenia of pregnancy are associated with hypertensive disorders. These disorders include the classifications preeclampsia, preeclampsia-eclampsia, preeclampsia with chronic hypertension, chronic hypertension, and gestational hypertension. Preeclampsia complicates about 5% of all pregnancies and typically occurs at about 20 weeks of gestation. The disorder is characterized by the onset of hypertension and proteinuria and may include abdominal pain, headache, blurred vision, or mental function disturbances.⁷⁷ Thrombocytopenia occurs in 15% to 20% of patients with preeclampsia, and about 40% to 50% of these patients progress to eclampsia (hypertension, proteinuria, and seizures).^{78,79}

Some patients with preeclampsia have microangiopathic hemolysis, elevated liver enzymes, and a low platelet count, termed *HELLP syndrome*. HELLP syndrome affects an estimated 4% to 12% of women with severe preeclampsia^{45,80,81} and seems to be associated with higher rates of maternal and fetal complications. This disorder may be difficult to differentiate from thrombotic thrombocytopenic purpura (TTP), hemolytic uremic syndrome (HUS), and disseminated intravascular coagulation (DIC).

The development of thrombocytopenia in these patients is thought to be due to increased platelet destruction. The mechanism of platelet destruction is unclear, however. Some evidence (elevated D-dimer) suggests that these patients have an underlying low-grade DIC.⁸² Elevated platelet-associated immunoglobulin is commonly found in these patients, however, which indicates immune involvement.⁸³ Early reports suggested that there may be a component of in vivo platelet activation because low-dose aspirin therapy has been shown to prevent preeclampsia in high-risk patients.^{84,85} When aspirin is used to prevent preeclampsia, however, reduction in risk is only 15%.

The treatment of preeclampsia is delivery of the infant whenever possible. After delivery, the thrombocytopenia usually resolves in a few days. In cases in which delivery is not possible (e.g., the infant would be too premature), bed rest and aggressive treatment of the hypertension may help to increase the platelet count in some patients. Such treatments include magnesium sulfate and other antiepileptic therapies to inhibit eclamptic seizures.

Other causes of thrombocytopenia during pregnancy.

As has been discussed previously, ITP is a relatively common disorder in women of childbearing age, and pregnancy does nothing to ameliorate the symptoms of this disorder. ITP

should be a part of the differential diagnosis of thrombocytopenia in a pregnant woman. There is little or no correlation between the level of maternal autoantibodies and the fetal platelet count. Other causes of thrombocytopenia during pregnancy include HIV infection, systemic lupus erythematosus, antiphospholipid syndromes, TTP, and HUS. Of all women who develop TTP, 10% to 25% manifest the disease during pregnancy or in the postpartum period, and TTP tends to recur in subsequent pregnancies.^{86,87} Plasmapheresis is the treatment of choice, and the maternal mortality is 90% or greater without such treatment.

Hemolytic Disease of the Newborn. Thrombocytopenia, usually moderate in degree, occurs frequently in infants with hemolytic disease of the newborn. Although the RBC destruction characteristic of this disorder is antibody induced, the antigens against which the antibodies are directed are not expressed on platelets. Platelets may be destroyed as a result of their interaction with products of RBC breakdown, rather than their direct participation in an immunologic reaction.³⁶

Thrombotic Thrombocytopenic Purpura. TTP, sometimes referred to as Moschcowitz syndrome, is characterized by the triad of microangiopathic hemolytic anemia, thrombocytopenia, and neurologic abnormalities.⁸⁸ In addition, fever and renal dysfunction (forming a pentad) are often present. Additional symptoms are present in most patients at the time of diagnosis and include diarrhea, anorexia, nausea, weakness, and fatigue. TTP is uncommon but not rare, and its incidence may be increasing. About twice as many women as men are affected, and it is most common in women 30 to 40 years of age.^{34,89} About half of the patients who develop TTP have a history of a viral-like illness several days before the onset of TTP.

There seem to be at least four types of TTP. In most patients, TTP occurs as a single acute episode, although a small fraction of these patients may have recurrence at seemingly random intervals. Recurrent TTP occurs in 11% to 28% of TTP patients.^{90,91} A third type of TTP is drug induced. The primary agents involved are the purinoreceptor (adenosine diphosphate) blocking agents ticlopidine (Ticlid) and clopidogrel (Plavix) used for inhibition of platelet function. Ticlopidine seems to cause TTP in about 0.025% of patients, whereas the incidence of clopidogrel-induced TTP is approximately four times less frequent.⁸⁹ The fourth type is chronic relapsing TTP, a rare form of TTP, in which episodes occur at intervals of approximately 3 months starting in infancy.^{92,93}

Although it is unclear what triggers their deposition, hyaline thrombi are found in the end arterioles and capillaries. These hyaline thrombi are composed of platelets and von Willebrand factor (VWF) but contain very little fibrin or fibrinogen. As these platelet-VWF thrombi are deposited, thrombocytopenia develops. The degree of thrombocytopenia is directly related to the extent of microvascular platelet aggregation. RBCs flowing under arterial pressure are prone to fragmentation and hemolysis when they encounter the strands of these thrombi.

Hemolysis is usually quite severe, and most patients have less than 10 g/dL hemoglobin at the time of diagnosis. Examination

of the peripheral blood film reveals a marked decrease in platelets, RBC polychromasia, and RBC fragmentation (microspherocytes, schistocytes, keratocytes), a triad of features characteristic of microangiopathic hemolytic anemias (Figure 40-8). Nucleated RBC precursors also may be present, depending on the degree of hemolysis. Other laboratory evidence of intravascular hemolysis includes reduction of haptoglobin, hemoglobinuria, hemosiderinuria, increased serum unconjugated bilirubin, and

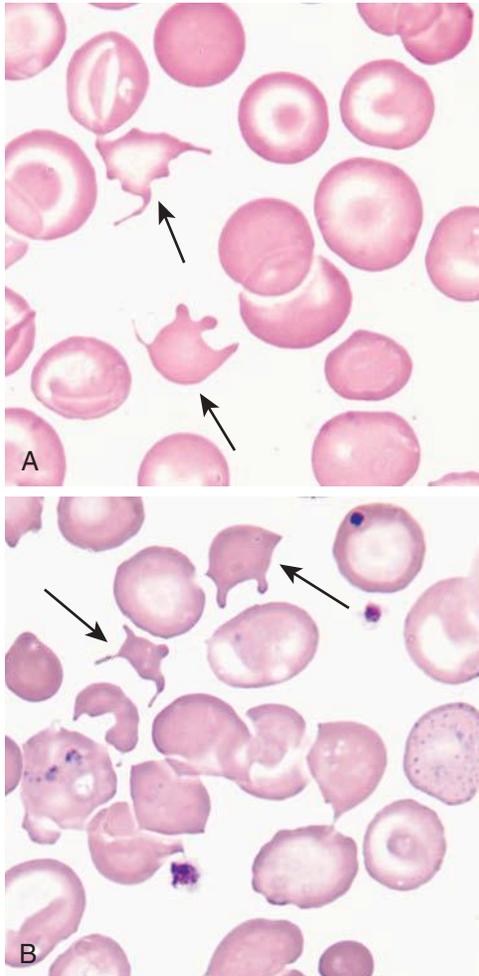


Figure 40-8 Microangiopathic hemolytic anemia. **A**, Thrombotic thrombocytopenic purpura (TTP); **B**, Hemolytic uremic syndrome (HUS). Abundant schistocytes (arrows) reflect the platelet rich clots in the microvasculature that occur with TTP and HUS. TTP and HUS have similar blood morphologies. (From Carr JH, Rodak BF: *Clinical hematology atlas*, ed 4, Philadelphia, 2013, Saunders.)

increased lactate dehydrogenase activity. Bone marrow examination reveals erythroid hyperplasia and a normal to increased number of megakaryocytes. The partial thromboplastin time, prothrombin time, fibrinogen, fibrin degradation products, and D-dimer test results are usually normal and may be useful in differentiating this disorder from DIC.

The thrombotic lesions also give rise to the other characteristic manifestations of TTP, because they are deposited in the vasculature of all organs. The thrombi occlude blood flow and lead to organ ischemia. Symptoms depend on the severity of ischemia in each organ. Neurologic manifestations range from headache to paresthesia and coma. Visual disturbances may be of neurologic origin or may be due to thrombi in the choroid capillaries of the retina or hemorrhage into the vitreous. Renal dysfunction is common and present in more than half of patients.^{90,91} Symptoms of renal dysfunction include proteinuria and hematuria. Overwhelming renal damage with anuria and fulminant uremia usually does not occur, however, which helps to distinguish TTP from HUS.¹⁰ Gastrointestinal bleeding occurs frequently in severely thrombocytopenic patients, and abdominal pain is occasionally present due to occlusion of the mesenteric microcirculation.

ADAMTS-13 and thrombotic thrombocytopenic purpura.

The development of TTP seems to be directly related to the accumulation of ultra-large von Willebrand factor (ULVWF) multimers in the plasma of patients with TTP. VWF multimers are made by megakaryocytes and endothelial cells. The primary source of plasma VWF seems to be endothelial cells. Endothelial cells secrete VWF into the subendothelium and plasma and store it in Weibel-Palade bodies (storage granules). Endothelial cells and megakaryocytes make even larger VWF multimers (ULVWF multimers) than those found in plasma, and these are even more effective than the normal plasma VWF multimers at binding platelet GP Ib/IX or GP IIb/IIIa complexes under fluid shear stresses (Figure 40-9).⁹² In the plasma, the ULVWF multimers are rapidly cleaved into the smaller VWF multimers normally found in the plasma by a VWF-cleaving protease, called *a* disintegrin and metalloprotease with a thrombospondin type 1 motif, member 13 (ADAMTS-13). This metalloprotease seems to be more effective when VWF multimers are partially unfolded by high shear stress.^{94,95}

Familial chronic relapsing TTP is a form characterized by recurrent episodes of thrombocytopenia with or without ischemic organ damage. In this type of TTP, the VWF-cleaving metalloprotease is completely deficient. The more common

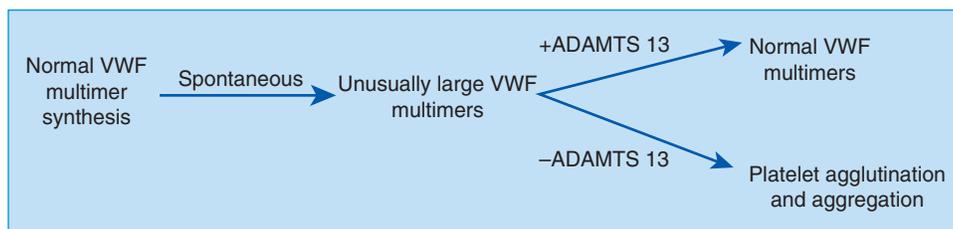


Figure 40-9 Mechanism for thrombotic thrombocytopenic purpura (TTP). Unusually large von Willebrand factor (ULVWF) multimers are normally digested by the VWF-cleaving protease ADAMTS-13 (a disintegrin and metalloprotease with a thrombospondin type 1 motif, member 13). In TTP, the absence of ADAMTS-13 allows the release of ULVWF, triggering platelet activation.

form of TTP (usually not familial) does not tend to recur, but patients also are deficient in the metalloprotease. In this more common form of TTP, the metalloprotease deficiency occurs through removal of the enzyme (or blockade of its function) by a specific autoantibody that is present during TTP but disappears during remission.^{96,97} An assay to measure the VWF-cleaving enzyme has been introduced.⁹⁸ However, the test may take several days, and treatment decisions must usually be made before test results are available. In addition, the assay lacks sensitivity and specificity for TTP. Additional tests for ADAMTS-13 (and TTP) are in development and hold the promise for rapid diagnosis of TTP.

In patients with TTP, ULVWF multimers tend to be present in the plasma at the beginning of the episode. These ULVWF multimers, and usually the normal-sized plasma multimers, disappear as the TTP episode progresses and the thrombocytopenia worsens. Platelets and VWF are consumed during deposition of the microvascular hyaline thrombi characteristic of this disorder.⁹⁹ If the patient survives an episode of TTP and does not experience relapse, the plasma VWF multimers are usually normal after recovery. If ULVWF multimers are found in the plasma after recovery, however, it is likely that the patient will have recurrent episodes of TTP. The episodes may be infrequent and at irregular intervals (intermittent TTP) or frequent and at regular intervals, as is often the case when TTP episodes occur in early childhood or infancy.

The most effective treatment for TTP is plasma exchange using fresh-frozen plasma or cryoprecipitate-poor plasma (plasma lacking most of the fibrinogen, fibronectin, and VWF).¹⁰⁰ Either of these approaches may produce dramatic effects within a few hours. Because plasmapheresis is not available in all centers, the patient should be given corticosteroids and infusions of fresh-frozen plasma immediately. Plasma exchange should be arranged as quickly as possible. Plasmapheresis and replacement/infusion of plasma is effective on two fronts. First, some of the ULVWF multimers will be removed by apheresis, and plasma (fresh-frozen plasma or cryoprecipitate-poor plasma) supplies the deficient protease, which is able to degrade the ULVWF multimers in the blood of the patient. Because some patients with TTP have recovered while receiving immunosuppressive treatment (corticosteroids) alone, it is recommended that all patients with TTP be given high-dose corticosteroids in addition to undergoing plasma exchange. Plasma exchange typically is continued over a 5-day period. If a response is not seen within 5 days, or if the condition of the patient worsens during the first few days of plasma exchange, additional treatment is instituted. Such therapies include administering vincristine or azathioprine (or other immunosuppressive agents), performing splenectomy, or passing the patient's plasma over a staphylococcal A column to remove immune complexes. The use of antiplatelet agents, prostacyclin, heparin, or fibrinolytic agents is controversial and has not clearly been shown to be helpful. Platelet transfusions should be avoided unless intracranial bleeding or other serious hemorrhagic problems arise.⁴⁵

Before 1990, TTP was fatal in more than 80% of patients. With the means for rapid diagnosis and the advent of exchange

plasmapheresis, now 80% of patients who are treated early can be expected to survive. Because patients are known to experience relapse, however, platelet counts should be monitored on a regular basis until patients are in remission. The detection of ULVWF multimers in patient samples after complete remission has predicted relapse accurately in 90% of the patients tested¹⁰¹; this may prove to be useful in the long-term management of TTP.

Hemolytic Uremic Syndrome. Clinically, HUS resembles TTP except that it is found predominantly in children 6 months to 4 years of age and is self-limiting. Approximately 90% of cases are caused by *Shigella dysenteriae* serotypes or enterohemorrhagic *Escherichia coli* OH serotypes, particularly O157:H7.¹⁰² These organisms sometimes can be cultured from stool samples. The bloody diarrhea typical of childhood HUS is caused by colonization of the large intestine with the offending organism, which causes erosive damage to the colon. *S. dysenteriae* produces Shiga toxin, and enterohemorrhagic *E. coli* produces either Shiga-like toxin-1 (SLT-1) or SLT-2, which can be detected in fecal samples from patients with HUS. The toxins enter the bloodstream and attach to renal glomerular capillary endothelial cells, which become damaged and swollen and release ULVWF multimers.^{102,103} This process leads to formation of hyaline thrombi in the renal vasculature and the development of renal failure, thrombocytopenia, and microangiopathic hemolytic anemia, although the RBC fragmentation is usually not as severe as that seen in TTP (this is, however, not a differentiating feature). The extent of renal involvement correlates with the rate of recovery. In more severely affected children, renal dialysis may be needed. The mortality rate associated with HUS in children is much lower than that for TTP, but there is often residual renal dysfunction that may lead to renal hypertension and severe renal failure. Because HUS in children is essentially an infectious disorder, it affects boys and girls equally and is often found in geographic clusters of cases rather than in random distribution.

The adult form of HUS is associated most often with exposure to immunosuppressive agents or chemotherapeutic agents or both, but it also may occur during the postpartum period. Usually the symptoms of HUS do not appear until weeks or months after exposure to the offending agent.¹⁰⁴ This disorder most likely results from direct renal arterial endothelial damage caused by the drug or one of its metabolic products. The damage to endothelial cells results in release of VWF (including ULVWF multimers), turbulent flow in the arterial system with increased shear stresses on platelets, and VWF-mediated platelet aggregation in the renal arterial system. The renal impairment in adults seems to be more severe than that in childhood HUS, and dialysis is usually required. The cause of HUS associated with pregnancy or oral contraceptive use is unclear, but it may be related to development of an autoantibody to endothelial cells. In outbreaks of HUS associated with consumption of *E. coli*-contaminated water, both children and adults have developed HUS.

The cardinal signs of HUS are hemolytic anemia, renal failure, and thrombocytopenia. The thrombocytopenia is usually mild to moderate in severity. Renal failure is reflected in

elevated blood urea nitrogen and creatinine levels. The urine nearly always contains RBCs, protein, and casts. The hemolytic process is shown by a hemoglobin level of less than 10 g/dL, elevated reticulocyte count, and presence of schistocytes in the peripheral blood.

Differentiating the adult form of HUS from TTP may be difficult. The lack of neurologic symptoms, the presence of renal dysfunction, and the absence of other organ involvement suggest HUS. Also, in HUS the thrombocytopenia tends to be mild to moderate (platelet consumption occurs primarily in the kidneys), whereas in TTP the thrombocytopenia is usually severe. Similarly, fragmentation of RBCs and the resultant anemia tend to be milder than that observed in TTP, because RBCs are being fragmented primarily in the kidneys. In some cases of HUS, other organs become involved, and the differentiation between HUS and TTP becomes less clear. In such cases, it is prudent to treat as though the patient has TTP.

Disseminated Intravascular Coagulation. A common cause of destructive thrombocytopenia is activation of the coagulation cascade (by a variety of agents or conditions), resulting in a consumptive coagulopathy that entraps platelets in intravascular fibrin clots. This disorder is described in more detail in Chapter 39 but is discussed here briefly for the sake of completeness. DIC has many similarities to TTP, including microangiopathic hemolytic anemia and deposition of thrombi in the arterial circulation of most organs. In DIC, however, the thrombi are composed primarily of platelets and fibrinogen, whereas in TTP the thrombi are composed primarily of platelets and VWF.

One form of DIC is acute with rapid platelet consumption and results in severe thrombocytopenia. In addition, levels of factor V, factor VIII, and fibrinogen are decreased as a result of in vivo thrombin generation. The test for D-dimer (a breakdown product of stabilized fibrin) almost always yields positive results. This form of DIC is life-threatening and must be treated immediately.

In chronic DIC, there is an ongoing, low-grade consumptive coagulopathy. Clotting factors may be slightly reduced or normal, and compensatory thrombocytopoiesis results in a moderately low to normal platelet count.³⁴ D-dimer may not be detectable or may be slightly to moderately increased. Chronic DIC is not generally life-threatening, and treatment usually is not urgent. Chronic DIC is almost always due to some underlying condition. If that condition can be corrected, the DIC usually resolves without further treatment. Chronic DIC should be followed closely, however, because it can convert into the life-threatening acute form.

Drug-Induced Thrombocytopenia. A few drugs directly interact with platelets to cause thrombocytopenia. Ristocetin, an antibiotic no longer in clinical use, facilitates the interaction of VWF with platelet membrane GP Ib and leads to in vivo platelet agglutination and thrombocytopenia. Hematin, used for the treatment of acute intermittent porphyria, may give rise to a transient thrombocytopenia that seems to be caused by stimulation of platelet secretion and aggregation. Protamine sulfate and bleomycin may induce thrombocytopenia by a similar mechanism.⁴⁵

Abnormalities in Distribution or Dilution

An abnormal distribution of platelets also may cause thrombocytopenia. The normal spleen sequesters approximately one third of the total platelet mass. Mild thrombocytopenia may be present in any of the “big spleen” syndromes. The total body platelet mass is often normal in these disorders, but numerous platelets are sequestered in the enlarged spleen, and consequently the venous blood platelet count is low. Disorders such as Gaucher disease, Hodgkin disease, sarcoidosis, lymphoma, cirrhosis of the liver, and portal hypertension may result in splenomegaly and lead to thrombocytopenia.

Lowering the body temperature to less than 25° C, as is routinely done in cardiovascular surgery, results in a transient but mild thrombocytopenia secondary to platelet sequestration in the spleen and liver. An associated transient defect in function also occurs with hypothermia. Platelet count and function return to baseline values on return to normal body temperature.³²

Thrombocytopenia often follows surgery involving extracorporeal circulatory devices, as a consequence of damage and partial activation of platelets in the pump. In a few cases, severe thrombocytopenia, marked impairment of platelet function, and activation of fibrinolysis and intravascular coagulation may develop.⁴⁵

The administration of massive amounts of stored whole blood may produce a temporary thrombocytopenia. This phenomenon is explained by the fact that stored blood contains platelets whose viability is severely impaired by the effects of storage and temperature. Under these conditions, the dead or damaged platelets are rapidly sequestered by the reticuloendothelial system of the patient. If this problem is encountered, it may be minimized by administering platelet concentrates or units of fresh whole blood along with the stored blood. This situation is only rarely encountered, however, because the practice of transfusing whole blood has been replaced almost completely by the use of specific components. Finally, mild thrombocytopenia may be encountered in patients with chronic renal failure, severe iron deficiency, megaloblastic anemia, postcompression sickness, and chronic hypoxia.

THROMBOCYTOSIS: INCREASE IN CIRCULATING PLATELETS

Thrombocytosis is defined as an abnormally high platelet count, typically more than 450,000/ μ L. The term *reactive thrombocytosis* is used to describe an elevation in the platelet count secondary to inflammation, trauma, or other underlying and seemingly unrelated conditions. In reactive thrombocytosis, the platelet count is elevated for a limited period and usually does not exceed 800,000/ μ L, although platelet counts greater than 1 million/ μ L are occasionally seen (Figure 40-10). A marked and persistent elevation in the platelet count is a hallmark of myeloproliferative disorders such as polycythemia vera, chronic myelogenous leukemia, and myelofibrosis with myeloid metaplasia (or primary myelofibrosis). In these conditions, the platelet count often exceeds 1 million/ μ L. Although the terms *thrombocythemia* and *thrombocytosis* are often used

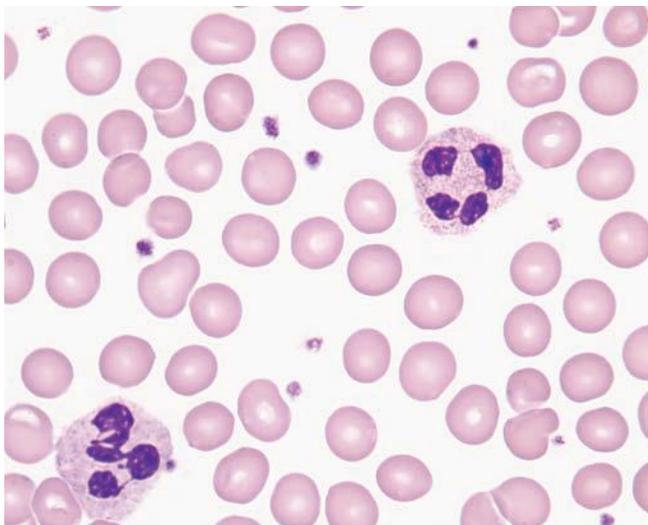


Figure 40-10 Peripheral blood film showing cell morphology in reactive thrombocytosis. Note the increased number of platelets but reasonably normal platelet morphology, characteristic of reactive thrombocytosis.

interchangeably, in this text the term *thrombocythemia* is used only as part of the description of the myeloproliferative disorder known as *essential thrombocythemia* (Figure 40-11). In essential thrombocythemia, platelet counts typically exceed 1 million/ μL and may reach levels of several million.^{34,105,106} Processes resulting in thrombocytosis are summarized in Box 40-3.

Reactive (Secondary) Thrombocytosis

Platelet counts between 450,000/ μL and 800,000/ μL with no change in platelet function can result from acute blood loss, splenectomy, childbirth, and tissue necrosis secondary to surgery, chronic inflammatory disease, infection, exercise, iron deficiency anemia, hemolytic anemia, renal disorders, and malignancy. Occasionally, patients manifest a platelet count of 1 to 2 million/ μL (Figure 40-10). In reactive thrombocytosis,

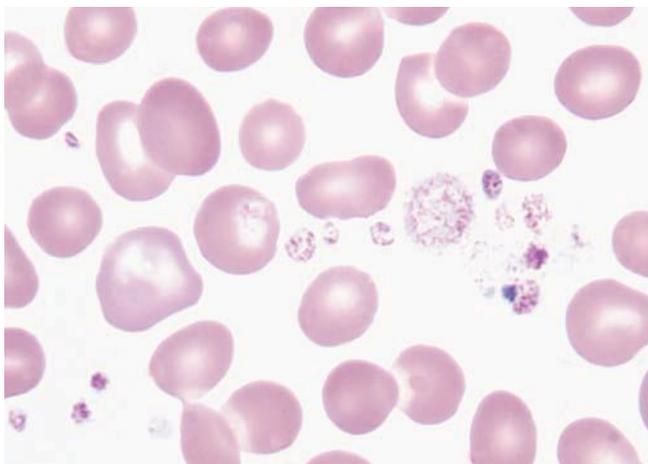


Figure 40-11 Peripheral blood film showing cell morphology in essential thrombocythemia. Note the increased number of platelets and wide variation in platelet size characteristic of essential thrombocythemia. Red and white blood cell morphology is characteristically normal. (From Carr JH, Rodak BF: *Clinical hematology atlas*, ed 4, Philadelphia, 2013, Saunders.)

BOX 40-3 Processes Resulting in Thrombocytosis

Conditions Associated with Reactive Thrombocytosis

- Blood loss and surgery
- Splenectomy
- Iron deficiency anemia
- Inflammation and disease
- Stress or exercise

Myeloproliferative Disorders Associated with Thrombocytosis

- Polycythemia vera
- Chronic myelogenous leukemia
- Myelofibrosis with myeloid metaplasia
- Thrombocythemia: essential or primary

From Colvin BT: Thrombocytopenia, *Clin Haematol* 14:661-681, 1985; and Thompson AR, Harker LA: *Manual of hemostasis and thrombosis*, ed 3, Philadelphia, 1983, FA Davis.

platelet production remains responsive to normal regulatory stimuli (e.g., thrombopoietin, a humoral factor that is produced in the kidney parenchyma), and morphologically normal platelets are produced at a moderately increased rate. This is in contrast to essential thrombocythemia, which is characterized by unregulated or autonomous platelet production and platelets of variable size.^{105,106}

Examination of the bone marrow from patients with reactive thrombocytosis reveals a normal to increased number of megakaryocytes that are normal in morphology. Results of platelet function tests, including aggregation induced by various agents, and bleeding time are usually normal in reactive thrombocytosis but also may be normal in patients with elevated platelet counts accompanying myeloproliferative disorders.

Reactive thrombocytosis is not associated with thrombosis, hemorrhage, or abnormal thrombopoietin levels. It seldom produces symptoms per se and disappears when the underlying disorder is brought under control.^{105,106}

Reactive Thrombocytosis Associated with Hemorrhage or Surgery

After acute hemorrhage, the platelet count may be low for 2 to 6 days (unless platelets have been transfused) but typically rebounds to elevated levels for several days before returning to the prehemorrhage level. A similar pattern of thrombocytopenia and thrombocytosis is seen after major surgical procedures in which there is significant blood loss. In both cases, the platelet count typically returns to normal 10 to 16 days after the episode of blood loss.

Postsplenectomy Thrombocytosis

Removal of the spleen typically results in platelet counts that can reach or exceed 1 million/ μL regardless of the reason for splenectomy. The spleen normally sequesters about one third of the circulating platelet mass. After splenectomy, one would

expect an initial increase in the platelet count of approximately 30% to 50%. The platelet count, however, far exceeds levels that could result from rebalancing of the circulating platelet pool to incorporate the splenic platelet pool. The cause of the accelerated platelet production is unknown. Unlike after blood loss from hemorrhage or other types of surgery, the platelet count reaches a maximum 1 to 3 weeks after splenectomy and remains elevated for 1 to 3 months. In some patients who undergo splenectomy for treatment of chronic anemia, the count can remain elevated for several years.

Thrombocytosis Associated with Iron Deficiency Anemia

Mild iron deficiency anemia secondary to chronic blood loss is associated with thrombocytosis in about 50% of cases. Thrombocytosis can be seen in severe iron deficiency anemia, but thrombocytopenia also has been reported. In some cases of iron deficiency, the platelet count may be 2 million/ μL . After iron therapy is started, the platelet count usually returns to normal within 7 to 10 days. It is believed that iron plays some role in regulating thrombopoiesis, because treatment of the iron deficiency with iron replacement has resulted in a normalization of the platelet count in thrombocytopenic patients and has been reported to induce thrombocytopenia in patients with normal platelet counts. Not enough research has been done, however, to elucidate the role of iron in thrombopoiesis.

Thrombocytosis Associated with Inflammation and Disease

Similar to elevations in C-reactive protein, fibrinogen, VWF, and other acute phase reactants, thrombocytosis may be an indication of inflammation. Thrombocytosis may be found in association with rheumatoid arthritis, rheumatic fever, osteomyelitis, ulcerative colitis, acute infections, and malignancy. In rheumatoid arthritis, the presence of thrombocytosis can be correlated with activation of the inflammatory process.

Kawasaki disease (Kawasaki syndrome) causes inflammation of the walls of small and medium-sized arteries throughout the body. It is also known as mucocutaneous lymph node syndrome because it affects lymph nodes, skin, and mucous membranes in the mouth, nose, and throat. It is an acute febrile illness of infants and young children. Boys are more likely than girls to develop the disease, and children of Japanese and Korean descent have higher rates of Kawasaki disease. It is a self-limited acute vasculitic syndrome of unknown origin, although an infectious etiology has been suspected. Although the disease is self-limiting, there can be lifelong sequelae, including coronary artery thrombosis and aneurysms. The acute febrile stage of the disease lasts 2 weeks or longer, with a fever of 40° C or higher, and is unresponsive to antibiotic therapy. The longer the fever continues, the higher the risk of cardiovascular complications. The subacute phase lasts an additional week to 10 days. During this phase, the platelet count usually is elevated, and counts of 2 million/ μL have been reported. In addition, acute phase reactants such as C-reactive protein and sedimentation rate are elevated, consistent with an inflammatory component. The WBC count can be moderately to markedly elevated with a

left shift, and many patients develop a mild normochromic, normocytic anemia. During this phase, cardiovascular complications and aneurysms develop. The higher the platelet count, the higher the risk of cardiovascular complication. After the subacute phase comes the convalescent phase, during which all signs of illness disappear and the acute phase reactants subside to normal. The highest incidence of Kawasaki disease is found in Japan and in individuals of Japanese descent, although the disease seems to occur in most, if not all, ethnic groups. There is no specific test for Kawasaki disease. Diagnosis is primarily by excluding other diseases that cause similar signs and symptoms (e.g., scarlet fever, juvenile rheumatoid arthritis, Stevens-Johnson syndrome, and toxic shock syndrome). The treatment for Kawasaki disease is administration of antiplatelet agents and immunoglobulin.

An elevated platelet count also may be early evidence of a tumor (e.g., Hodgkin disease) and various carcinomas. Finally, hemophilic patients often have platelet counts above normal limits, even in the absence of active bleeding.

Exercise-Induced Thrombocytosis

Strenuous exercise is a well-known cause of relative thrombocytosis and is likely due to the release of platelets from the splenic pool or hemoconcentration by transfer of plasma water to the extravascular compartment or both. Normally, the platelet count returns to its preexercise baseline level 30 minutes after completion of exercise.

Rebound Thrombocytosis

Thrombocytosis often follows the thrombocytopenia caused by marrow-suppressive therapy or other conditions. "Rebound" thrombocytosis usually reaches a peak 10 to 17 days after withdrawal of the offending drug (e.g., alcohol or methotrexate) or after institution of therapy for the underlying condition with which thrombocytopenia is associated (e.g., vitamin B₁₂ deficiency).⁴⁵

Thrombocytosis Associated with Myeloproliferative Disorders

Primary or autonomous thrombocytosis is a typical finding in four chronic myeloproliferative disorders: polycythemia vera, chronic myelogenous leukemia, myelofibrosis with myeloid metaplasia (primary myelofibrosis), and essential thrombocythemia. Depending on the duration and stage of the myeloproliferative disorder at the time of diagnosis, it may be difficult to differentiate among these diseases. Chapter 33 provides a more complete description of these disorders. In the other types of myeloproliferative disorders, the platelet count seldom reaches the extreme values characteristic of essential thrombocythemia. Diagnosis of essential thrombocythemia should not be based on the platelet count alone but should also take into account the physical examination findings, history, and other laboratory data.^{34,106}

Essential (Primary) Thrombocythemia

Essential thrombocythemia is a clonal disorder related to other chronic myeloproliferative diseases and is the most common

cause of primary thrombocytosis. It is characterized by peripheral blood platelet counts exceeding 1 million/ μL and uncontrolled proliferation of marrow megakaryocytes. Although the platelet count may (or may not) be markedly elevated in other myeloproliferative disorders, persistent marked elevation of the platelet count is an absolute requirement for the diagnosis of essential thrombocythemia (Figure 40-11). There is evidence that essential thrombocythemia is caused by a clonal proliferation of a single abnormal pluripotential stem cell that eventually crowds out normal stem cells. As with most myeloproliferative disorders, essential thrombocythemia is neither congenital nor hereditary, is prevalent in middle-aged and older patients, and affects equal numbers of men and women. In contrast to other myeloproliferative disorders, however, the other marrow cell lines are not involved at the time of diagnosis.

The clinical manifestations of essential thrombocythemia are hemorrhage, platelet dysfunction, and thrombosis. Bleeding times are usually normal. There is no specific clinical sign, symptom, or laboratory test that establishes the diagnosis of essential thrombocythemia. The diagnosis must be made by ruling out the other myeloproliferative disorders and systemic illnesses that produce reactive thrombocytosis.

Thrombosis in the microvasculature is relatively common in essential thrombocythemia, and the incidence at the time of diagnosis is 10% to 20%. This thrombosis can lead to digital pain, digital gangrene, or erythromelalgia (throbbing, aching, and burning sensation in the extremities, particularly in the palms and soles).¹⁰⁶ The symptoms of erythromelalgia can be explained by arteriolar inflammation and occlusive thrombosis mediated by platelets and can be relieved for several days by a single dose of aspirin.¹⁰⁷ Thrombosis of large veins and arteries also may occur in essential thrombocythemia. The arteries most commonly involved are those in the legs, the coronary arteries, and the renal arteries, but involvement of the mesenteric, subclavian, and carotid arteries is not infrequent (in fact, neurologic complications are relatively common). Venous thrombosis may involve the large veins of the legs and pelvis, hepatic veins, or splenic veins.¹⁰⁸ The platelets of some patients who have experienced thrombotic episodes have been shown to have increased binding affinity for fibrinogen and to generate more than the usual quantities of thromboxane B_2 , and these patients have elevated levels of thromboxane B_2 and β -thromboglobulin in the blood. These findings suggest enhanced *in vivo* platelet activation and perhaps an explanation for the thrombotic tendencies of patients with essential thrombocythemia. The primary cause of death of patients with essential thrombocythemia seems to be thrombosis. Hemorrhagic episodes occur less frequently than thrombotic episodes in patients with essential thrombocythemia.

As with bleeding secondary to platelet function disorders, the hemorrhagic manifestations of essential thrombocythemia are mucocutaneous in nature, with gastrointestinal tract bleeding occurring most frequently. Other sites of bleeding include the mucous membranes of the nose and mouth, the urinary tract, and the skin. Symptoms may be aggravated by aspirin use. In an occasional patient with essential thrombocythemia, there is a paradoxical combination of thromboembolic (clotting) and hemorrhagic episodes in association with this condition.

A patient with essential thrombocythemia who has had a thrombotic event may have a hemorrhagic event later.¹⁰⁹

The bleeding manifestations may be related to a variety of qualitative abnormalities in the platelets, including deficiencies in epinephrine receptors and ultrastructural defects in granules, mitochondria, and microfilaments. Platelets may be agranular or hypogranular and have a clear, light blue appearance on a routine Wright-stained film of the peripheral blood. Although platelet size is heterogeneous, giant and bizarrely shaped platelets are characteristic of myeloproliferative diseases, and megakaryocyte fragments or nuclei are commonly encountered in the peripheral blood. Platelets may be notably clumped on blood films, exhibiting marked variation in size and shape. The number and volume of megakaryocytes are increased in the bone marrow, and they are predominantly large, show some cellular atypia, and tend to form clusters. Often the platelets are functionally defective when tested *in vitro*. Aggregation is usually absent in response to epinephrine and may be decreased with adenosine diphosphate but is usually normal with collagen. Lack of an epinephrine response may help to differentiate essential thrombocythemia from reactive thrombocytosis, because this response is usually normal in reactive thrombocytosis but absent in most cases of essential thrombocythemia. Platelet adhesion also may be decreased.¹⁰⁶

The degree of thrombocytosis has not been found to predict hemorrhagic or thrombotic events reliably. The role of lowering platelet counts as a prophylactic treatment in this disease is not established. The risks from exposure to mutagenic alkylating agents used to decrease the platelet count may be greater than the risk of thrombosis or hemorrhage. When treatment is deemed necessary because of thrombotic tendencies or splenomegaly, a variety of myelosuppressive agents (e.g., melphalan, busulfan, hydroxyurea, or even radioactive phosphorus) can be used.¹⁰⁶ In patients with life-threatening hemorrhage or thrombosis and an extremely high platelet count, platelet apheresis may be used to reduce the platelet count rapidly. In these situations, a myelosuppressive agent is added for longer-term control of the platelet count.¹¹⁰ Interferon- α has been used to treat essential thrombocythemia and is associated with an approximately 60% rate of complete remission, but 28% of patients given the drug cannot tolerate the dosages required.¹¹¹⁻¹¹³ A newer agent that has shown great promise in the treatment of essential thrombocythemia is anagrelide. The drug acts by inhibiting megakaryocyte maturation and platelet release.¹¹⁴ In one large study, anagrelide decreased the platelet count in 93% of patients.¹¹⁵ Many patients cannot tolerate anagrelide, however, and in these patients other, more traditional chemotherapeutic agents seem to be more effective. Despite the availability of newer treatments for essential thrombocythemia, whether a patient with essential thrombocythemia and an elevated platelet count who is asymptomatic should or should not be treated remains controversial.

In patients with essential thrombocythemia there is a low incidence of transformation to acute leukemia or fatal thrombotic or hemorrhagic complications. Therapy to prevent thrombotic complications seems to be effective in preventing morbidity but does not seem to improve survival, at least in high-risk patients.

SUMMARY

- Thrombocytopenia is the most common cause of clinically significant bleeding.
- Thrombocytopenia may be a result of decreased platelet production, increased destruction, or abnormal distribution of platelets and manifests with small-vessel bleeding in the skin.
- Decreased production of platelets can be attributed to megakaryocyte hypoplasia, ineffective thrombopoiesis, or replacement of marrow by abnormal cells.
- Patients experiencing increased platelet destruction become thrombocytopenic only when the rate of platelet production can no longer increase enough to compensate.
- Pathologic destruction of platelets can be caused by both immunologic and nonimmunologic mechanisms.
- Acute ITP commonly occurs in children after a viral illness, and there is usually spontaneous remission. Chronic ITP is more commonly seen in women and requires treatment if the platelet count decreases to fewer than 30,000/ μ L.
- Treatment of drug-induced thrombocytopenia must begin with identification of the causative drug and discontinuation of its use.
- TTP presents with a triad of symptoms that includes microangiopathic hemolytic anemia, thrombocytopenia, and neurologic abnormalities; these may be accompanied by fever and renal dysfunction.
- Abnormal distribution of platelets can be caused by splenic sequestration.
- Reactive thrombocytosis is secondary to inflammation, trauma, or a variety of underlying conditions. Platelet counts are increased for a limited time. The thrombocytosis seen in myeloproliferative disorders is marked and persistent.

Now that you have completed this chapter, go back and read again the case study at the beginning and respond to the questions presented.

REVIEW QUESTIONS

Answers can be found in the Appendix.

1. The autosomal dominant disorder associated with decreased platelet production is:
 - a. Fanconi anemia
 - b. TAR syndrome
 - c. May-Hegglin anomaly
 - d. Wiskott-Aldrich anomaly
2. Which of the following is *not* a hallmark of ITP?
 - a. Petechiae
 - b. Thrombocytopenia
 - c. Large overactive platelets
 - d. Megakaryocyte hypoplasia
3. The specific antigen most commonly responsible for the development of NAIT is:
 - a. Bak
 - b. HPA-1a
 - c. GP Ib
 - d. Lewis antigen a
4. A 2-year-old child with an unexpected platelet count of 15,000/ μ L and a recent history of a viral infection most likely has:
 - a. HIT
 - b. NAIT
 - c. Acute ITP
 - d. Chronic ITP
5. What is the first step in the treatment of HIT?
 - a. Start low-molecular-weight heparin therapy
 - b. Stop heparin infusion immediately
 - c. Switch to warfarin (Coumadin) immediately
 - d. Initiate a platelet transfusion
6. A defect in primary hemostasis (platelet response to an injury) often results in:
 - a. Musculoskeletal bleeding
 - b. Mucosal bleeding
 - c. Hemarthroses
 - d. None of the above
7. When a drug acts as a hapten to induce thrombocytopenia, an antibody forms against which of the following?
 - a. Typically unexposed, new platelet antigens
 - b. The combination of the drug and the platelet membrane protein to which it is bound
 - c. The drug alone in the plasma, but the immune complex then binds to the platelet membrane
 - d. The drug alone, but only when it is bound to the platelet membrane
8. TAR refers to:
 - a. Abnormal platelet morphology in which the radial striations of the platelets are missing
 - b. Abnormal appearance of the iris of the eye in which radial striations are absent
 - c. Abnormal bone formation, including hypoplasia of the forearms
 - d. Neurologic defects affecting the root (radix) of the spinal nerves

9. Neonatal autoimmune thrombocytopenia occurs when:
 - a. The mother lacks a platelet antigen that the infant possesses, and she builds antibodies to that antigen, which cross the placenta
 - b. The infant develops an autoimmune process such as ITP secondary to in utero infection
 - c. The infant develops an autoimmune disease such as lupus erythematosus before birth
 - d. The mother has an autoimmune antibody to her own platelets, which crosses the placenta and reacts with the infant's platelets
10. HUS in children is associated with:
 - a. Diarrhea caused by *Shigella* species
 - b. Meningitis caused by *Haemophilus* species
 - c. Pneumonia caused by *Mycoplasma* species
 - d. Pneumonia caused by respiratory viruses

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Qualitative Disorders of Platelets and Vasculature

Larry D. Brace

OUTLINE

Qualitative Platelet Disorders

Disorders of Platelet Aggregation Affecting GP IIb/IIIa Function

Disorders of Platelet Adhesion Affecting GP Ib/IX/V Complexes
Inherited Giant Platelet Syndromes

Disorders of Platelet Secretion (Release Reactions)

Inherited Disorders of Other Receptors and Signaling Pathways

Acquired Defects of Platelet Function

Vascular Disorders

Hereditary Vascular Disorders
Acquired Vascular Disorders

OBJECTIVES

After completion of this chapter, the reader will be able to:

1. Describe the effect of aspirin on the cyclooxygenase pathway.
2. Describe the defect in each of the following hereditary disorders: storage pool disease, gray platelet syndrome, Glanzmann thrombasthenia, and Bernard-Soulier syndrome (BSS).
3. Discuss the mechanisms of action of antiplatelet drugs.
4. Explain the effects of paraproteins on platelet function.
5. Compare and contrast Glanzmann thrombasthenia and BSS.
6. Recognize the clinical presentation of patients with dysfunctional platelets.
7. Distinguish among the following types of inherited platelet disorders: membrane receptor abnormality, secretion disorder, and storage pool deficiency.
8. For each of the inherited platelet disorders listed, name useful laboratory tests and recognize diagnostic results.
9. Identify the most common type of hereditary platelet defect.
10. Discuss the mechanism of the platelet defects associated with myeloproliferative diseases, uremia, and liver disease.
11. Recognize conditions associated with acquired vascular disorders.

CASE STUDY

After studying the material in this chapter, the reader should be able to respond to the following case study:

A 19-year-old woman with a chief complaint of easy bruising, occasional mild nosebleeds, and heavy menstrual periods was examined by her physician. At the time of her examination, she had a few small bruises on her arms and legs, but no other problems. Initial laboratory data revealed a normal prothrombin time, and a normal partial thromboplastin time. A CBC, including platelet count and morphology, yielded normal results.

A detailed history revealed that the patient's bleeding problems occurred most frequently after aspirin ingestion. Her mother and one of her brothers also had some of the same symptoms. Blood was drawn for platelet function studies, and platelet aggregation tests were performed. Aggregation induced by ristocetin and adenosine diphosphate was near normal, arachidonic acid-induced aggregation was absent, epinephrine induced only primary aggregation,

and collagen-induced aggregation was decreased, although a near-normal aggregation response could be obtained with a high collagen concentration. Spontaneous aggregation was not observed.

1. What are three possible explanations for the test results so far?
2. Given the bleeding history in her family, which of the three explanations seems most likely?

A quantitative test for adenosine triphosphate (ATP) release was performed using the firefly luciferin-luciferase bioluminescence assay. The result of this test showed a marked decrease in the amount of ATP released when platelets were stimulated with thrombin.

3. Based on the ATP test results, what is the likely cause of the patient's bleeding symptoms?

Clinical manifestations of bleeding disorders can be divided into two broad, rather poorly defined groups: (1) superficial bleeding (e.g., petechiae, epistaxis, or gingival bleeding), usually associated with a platelet defect or vascular disorder; and (2) deep tissue bleeding (e.g., hematomas or hemarthrosis), usually associated with plasma clotting factor deficiencies.¹ This chapter describes platelet and vascular disorders. Bleeding problems resulting from defects in the coagulation mechanism are described in Chapter 38.

QUALITATIVE PLATELET DISORDERS

Excessive bruising, superficial (mucocutaneous) bleeding, and a prolonged bleeding time in a patient whose platelet count is normal suggest an acquired or a congenital disorder of platelet function. Congenital disorders have been described that result from abnormalities of each of the major phases of platelet function: adhesion, aggregation, and secretion. Rapid progress in this field began in the 1960s, mostly as a result of the development of instruments and test methods for measuring platelet function.²

Qualitative platelet disorders are summarized in Box 41-1.^{3,4} This chapter discusses the individual qualitative platelet disorders grouped by the mechanism that causes the defect. A summary of the primary disorders is illustrated in Figure 41-1 for defects associated with surface components and Figure 41-2 for defects associated with intracellular components.⁵

Disorders of Platelet Aggregation Affecting GP IIb/IIIa Function

Glanzmann Thrombasthenia

Glanzmann thrombasthenia originally was described as a bleeding disorder associated with abnormal in vitro clot retraction and a normal platelet count. Clot retraction is the process of the compaction of a formed clot (reducing its volume), mediated by contraction of the intracellular actin-myosin cytoskeleton of the activated platelets incorporated in the clot. It is inherited as an autosomal recessive disorder and is seen most frequently in populations with a high degree of consanguinity. Heterozygotes are clinically normal, whereas homozygotes have serious bleeding problems. This rare disorder manifests itself clinically in the neonatal period or infancy, occasionally with bleeding after circumcision and frequently with epistaxis and gingival bleeding. Hemorrhagic manifestations include petechiae, purpura (Figure 40-1), menorrhagia, gastrointestinal bleeding, and hematuria. There are wide variations in the clinical symptoms. Some patients may have minimal symptoms, whereas others may have frequent and serious hemorrhagic complications. The severity of the bleeding episodes seems to decrease with age.^{6,7}

The biochemical lesion responsible for the disorder is a deficiency or abnormality of the platelet membrane glycoprotein (GP) IIb/IIIa (integrin α_{IIb}/β_3) complex, a membrane receptor capable of binding fibrinogen, von Willebrand factor (VWF), fibronectin, and other adhesive ligands. Typically, the platelets of homozygous individuals lack surface-expressed GP IIb/IIIa, whereas the GP IIb/IIIa content of the platelets from

BOX 41-1 Qualitative Abnormalities: Changes in Platelet Function (Thrombocytopathy)^{3,4}

Disorders of Platelet Aggregation

Glanzmann thrombasthenia
Hereditary afibrinogenemia
Acquired defects of platelet aggregation:
 Acquired von Willebrand disease
 Acquired uremia

Disorders of Platelet Adhesion

Bernard-Soulier syndrome
Von Willebrand disease
Acquired defects of platelet adhesion:
 Myeloproliferative, lymphoproliferative disorders, dysproteinemias
 Antiplatelet antibodies
 Cardiopulmonary bypass surgery
 Chronic liver disease
 Drug-induced membrane modification

Disorders of Platelet Secretion (release reactions)

Storage pool diseases
Thromboxane pathway disorders
Hereditary aspirin-like defects:
 Cyclooxygenase or thromboxane synthetase deficiency
 Drug inhibition of the prostaglandin pathways
 Drug inhibition of platelet phosphodiesterase activity

Changes in Membrane Phospholipid Distribution

Scott syndrome
Stormorken syndrome

Hyperactive Prothrombotic Platelets

heterozygotes has been found to be 50% to 60% of normal.^{8,9} Binding of fibrinogen to the GP IIb/IIIa complex mediates normal platelet aggregation responses. Failure of such binding results in a profound defect in hemostatic plug formation and the serious bleeding characteristic of thrombasthenia.^{2,8,10-13}

More than 70 mutations are known to give rise to Glanzmann thrombasthenia.¹⁴⁻¹⁶ GP IIb/IIIa is coded by the *ITGA2B* and *ITGB3* genes present on chromosome 17, and genetic defects are distributed widely over the two genes.¹⁷ Rarely, thrombocytopenia and large platelets may be seen with some mutations in these genes (Table 40-1). α_{IIb} is synthesized in megakaryocytes as pro- α_{IIb} , which complexes β_3 in the endoplasmic reticulum. The complex is transported to the Golgi body, where α_{IIb} is cleaved to heavy and light chains to form the complete complex. Uncomplexed α_{IIb} and β_3 are not processed in the Golgi body. As with the GP Ib/IX/V complex, it is necessary for both proteins of the GP IIb/IIIa complex to be produced and assembled into a complex for the complex to be expressed on the platelet surface. Gene defects that lead to the absence of

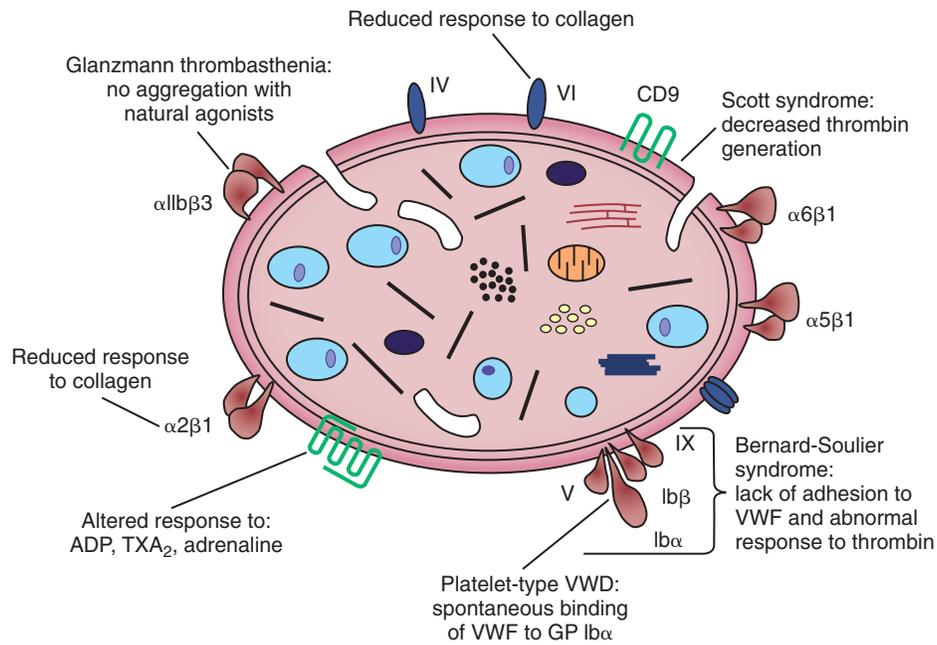


Figure 41-1 An illustration of the primary disorders associated with defects of the surface components of platelets. (From Nurden P and Nurder AT. Congenital disorders associated with platelet dysfunction. *Thromb Haemost* 2008;99:253-263.)

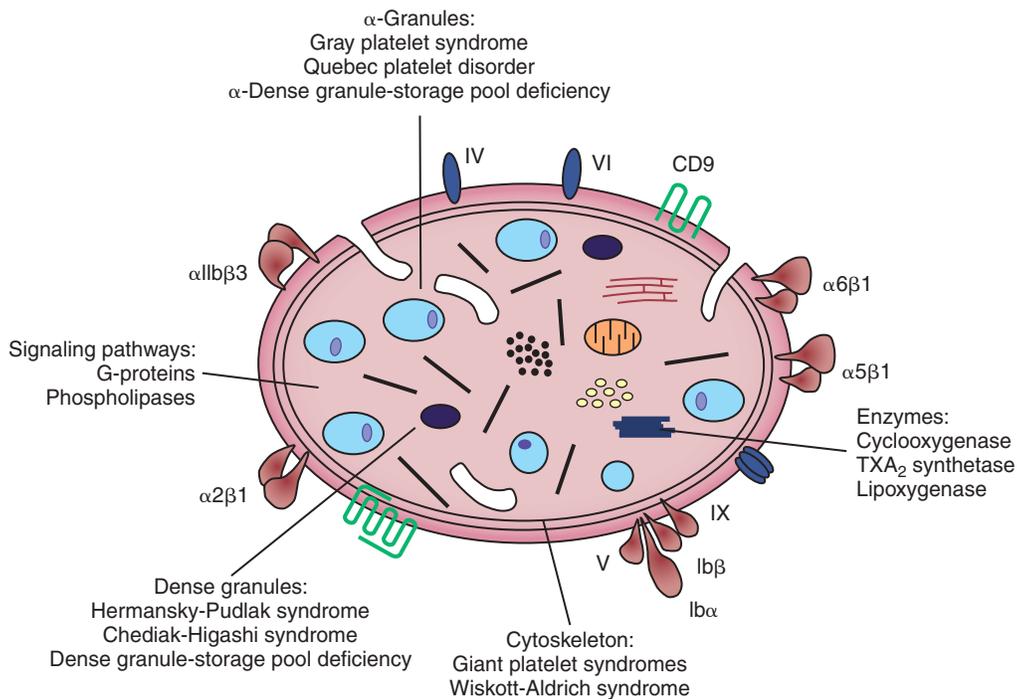


Figure 41-2 An illustration of the primary disorders associated with defects of the intracellular components of platelets. (From Nurden P and Nurder AT. Congenital disorders associated with platelet dysfunction. *Thromb Haemost* 2008;99:253-263.)

production of either protein lead to absence of the complex on the platelet surface. Defects that interfere with or prevent complex formation or affect complex stability have the same effect.

Numerous variants of Glanzmann thrombasthenia have been described in which the GP IIb/IIIa complex is qualitatively abnormal. α_{IIb} and β_3 are produced, form a complex, and are processed normally. One or more functions of the

complex (e.g., fibrinogen binding or signal transduction) are abnormal, however. Bleeding in these patients ranges from mild to severe.

One component of the α_{IIb}/β_3 integrin, β_3 , is a component of the vitronectin receptor, α_V/β_3 , found on endothelial cells, osteoclasts, fibroblasts, monocytes, and activated B lymphocytes, where it acts as a receptor for a variety of

adhesive protein ligands. Patients who have β_3 gene defects that result in the absence of α_{IIb}/β_3 integrin also lack the vitronectin receptor. These patients do not seem to have a more severe form of Glanzmann thrombasthenia.^{18,19} The vitronectin receptor is thought to play a role in vascularization, but so far, no evidence for abnormal blood vessel development has been documented in individuals lacking the vitronectin receptor. It also is unclear whether platelet vitronectin receptors play any significant role in platelet functional processes.¹⁴

Rarely, a thrombasthenia-like state can be acquired. Such conditions include development of autoantibodies against GP IIb/IIIa, multiple myeloma in which the paraprotein is directed against GP IIIa, and afibrinogenemia. A thrombasthenia-like state also can be induced in individuals with otherwise normal platelet function by the therapeutic antiplatelet drugs ticlopidine and clopidogrel.^{7,12}

Laboratory Features. The typical laboratory features of Glanzmann thrombasthenia are a normal platelet count, normal platelet morphology, and a lack of platelet aggregation in response to all platelet activating agents (including ADP, collagen, thrombin, and epinephrine).^{2,8,10,12} If the stimulating agent is strong enough (e.g., thrombin), the platelets undergo the release/secretion reaction, even in the absence of aggregation. Ristocetin-induced binding of VWF to platelets and the resulting platelet agglutination are normal. The results of the complete blood count (CBC) are usually normal, unless there is another underlying disorder or the patient has had a recent hemorrhagic episode. Tests for platelet procoagulant activity, previously called the platelet factor 3 test, usually show diminished activity.^{2,9,20} There seem to be several reasons for this. When normal platelets are activated, procoagulant microvesicles are shed from the platelet surface, and coagulation factors assemble on the microvesicle surfaces during activation of the coagulation cascade. In Glanzmann thrombasthenia, markedly fewer microvesicles are produced. Second, prothrombin binds directly to GP IIb/IIIa. Because this complex is missing in Glanzmann thrombasthenia, significantly less thrombin is generated in response to tissue factor. Finally, Glanzmann thrombasthenia platelets are not as activated by thrombin as are normal platelets.²¹⁻²⁴

A subdivision of Glanzmann thrombasthenia into type 1 and type 2 has been proposed. In general, individuals with type 2 disease have more residual GP IIb/IIIa complexes (10% to 20% of normal) than those with type 1 disease (0% to 5% of normal), although there is considerable variability within each subdivision.^{25,26}

Treatment. Thrombasthenia is one of the few forms of platelet dysfunction in which hemorrhage is severe and disabling. Bleeding of all types, including epistaxis, ecchymosis, hemarthrosis, subcutaneous hematoma, menorrhagia, and gastrointestinal and urinary tract hemorrhage, has been reported. Treatment of bleeding episodes in patients with Glanzmann thrombasthenia requires the transfusion of normal platelets. In Glanzmann thrombasthenia, the defective

platelets may interfere with the normal transfused platelets, and it may be necessary to infuse more donor platelets than expected to control bleeding. As in Bernard-Soulier syndrome or any situation in which repeated transfusions are required, patients with Glanzmann thrombasthenia may become alloimmunized. Strategies to reduce alloimmunization include use of single-donor platelet apheresis products, HLA-matched donor platelets, or ABO-matched donor platelets.²⁷

A variety of treatments have been used successfully to control or prevent bleeding, alone or in combination with platelet transfusion. To a large extent, the site of hemorrhage determines the therapeutic approach used. Hormonal therapy (norethindrone acetate) has been used to control menorrhagia. If the patient is treated with oral contraceptives, excessive bleeding should be reduced. Menorrhagia at the onset of menses is uniformly severe and can be life-threatening, which has led some to suggest that birth control pills be started before menarche. Also, antifibrinolytic therapy (aminocaproic acid or tranexamic acid) can be used to control gingival hemorrhage or excessive bleeding after tooth extraction.²⁶ Recombinant factor VIIa has proved useful to treat severe bleeding in patients with isoantibodies to α_{IIb}/β_3 and in patients undergoing invasive procedures.²⁸ Recombinant factor VIIa (rVIIa, NovoSeven, Novo Nordisk Inc, Princeton, NJ) is thought to enhance thrombus formation at the site of a lesion by stimulating tissue factor-independent thrombus generation and fibrin formation.²⁹

Disorders of Platelet Adhesion Affecting GP Ib/IX/V Complexes

Bernard-Soulier (Giant Platelet) Syndrome

Bernard-Soulier syndrome (BSS) is a rare disorder of platelet adhesion that is usually manifested in infancy or childhood with hemorrhage characteristic of defective platelet function: ecchymoses, epistaxis, and gingival bleeding. Hemarthroses and expanding hematomas are only rarely seen. BSS is inherited as an autosomal recessive disorder in which the GP Ib/IX/V complex is missing from the platelet surface or exhibits abnormal function. Heterozygotes who have about 50% of normal levels of GP Ib, GP V, and GP IX have normal or near-normal platelet function. Homozygotes have a moderate to severe bleeding disorder characterized by enlarged platelets, thrombocytopenia, and usually decreased platelet survival. Platelet counts generally range from 40,000/ μ L to near normal.³⁰ On peripheral blood films, platelets typically are 5 to 8 μ m in diameter, but they can be 20 μ m (Figure 41-3). Viewed by electron microscopy, BSS platelets contain a larger number of cytoplasmic vacuoles and membrane complexes, and these observations extend to megakaryocytes, in which the appearance of the demarcation membrane system is irregular.^{2,3,8,10,31}

Four glycoproteins are required to form the GP Ib/IX/V complex: GP Ib α , GP Ib β , GP IX, and GP V. In the complex, these proteins are present in the ratio of 2:2:2:1. The gene for GP Ib α is located on chromosome 17, the gene for GP Ib β is located on chromosome 22, and the genes for GP IX and GP V

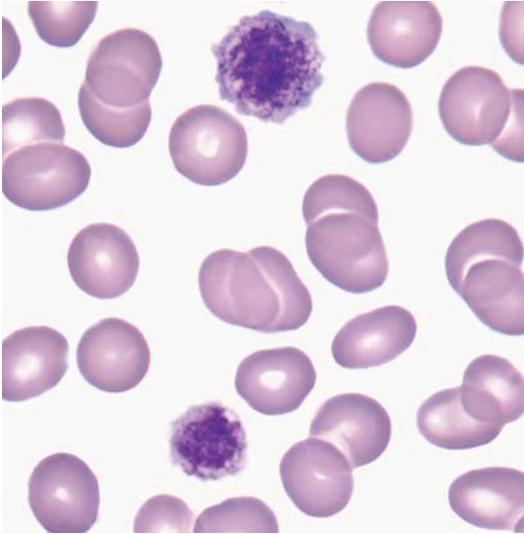


Figure 41-3 Giant platelets in Bernard-Soulier syndrome (peripheral blood, $\times 1000$). (Modified from Carr JH, Rodak BF: *Clinical hematology atlas*, ed 3, Philadelphia, 2009, Saunders.)

are located on chromosome 3. For surface expression of the GP Ib/IX complex, it seems that synthesis of three proteins, GP Ib α , GP Ib β , and GP IX, is required. Only GP V can be expressed alone in significant quantities on the surface of platelets, but the expression seems to be enhanced if the rest of the complex is present. The most frequent forms of BSS involve defects in GP Ib α synthesis or expression (Table 40-1). The presence of GP Ib α is essential to normal function because it contains binding sites for VWF and thrombin. Defects in the GP Ib β and GP IX genes also are known to result in BSS.³²⁻³⁴ Diseases causing mutations include missense, frameshift, and nonsense mutations.^{35,36}

Variants. Several unusual variants of BSS have been described in which all or most of the GP Ib/IX/V complex is present, but mutations that affect binding domains affect interactions between elements of the complex, or result in truncation of a specific protein in the complex. In these cases, the complex fails to bind VWF or does so poorly.³³ In rare circumstances, an antibody to GP Ib/V can cause a Bernard-Soulier–like syndrome (*pseudo-BSS*), in which the GP Ib/IX/V complex is nonfunctional.

Mutations in the GP Ib/IX/V complex can also give rise to gain of function and result in *platelet-type VWD (pseudo-VWD)*. This gain of function results in spontaneous binding of plasma VWF to the mutated GP Ib/IX/V complex. As a consequence, platelets and large VWF multimers with their associated factor VIII are removed from the circulation, resulting in thrombocytopenia and reduced factor VIII clotting activity. GP Ib α mutations that give rise to platelet-type VWD are 233 Gly \rightarrow Val or Ser and 239 Met \rightarrow Val.^{37,38} Loss of residues 421 to 429 in GP Ib α also has been reported to result in platelet-type VWD.³⁹

Laboratory Features. BSS platelets have normal aggregation responses to adenosine diphosphate (ADP), epinephrine, collagen, and arachidonic acid but do not respond to

ristocetin and have diminished response to thrombin.^{2,8,10} The lack of response to ristocetin is due to the lack of GP Ib/IX/V complexes and the inability of BSS platelets to bind VWF. Lack of binding to VWF also accounts for the inability of platelets to adhere to exposed subendothelium and the resultant bleeding characteristic of this disorder. This defect in adhesion shows the importance of initial platelet attachment in primary hemostasis. In many respects, this disorder resembles the defect seen in von Willebrand disease (VWD). In contrast to VWD, however, this abnormality cannot be corrected by the addition of normal plasma or cryoprecipitate, which is consistent with a defect that resides in the platelets.

Treatment. There is no specific treatment for BSS. Platelet transfusions are the therapy of choice, but patients invariably develop alloantibodies, so further platelet transfusion is not possible. BSS patients tend to do better if apheresis platelets are used for transfusion because this tends to limit the number of donors to which the patient is exposed, and the rate of alloimmunization tends to be lower.^{8,10} Other treatments that have been used include desmopressin acetate (DDAVP) and, more recently, recombinant factor VIIa (rVIIa, NovoSeven, Novo Nordisk Inc, Princeton, NJ).

Inherited Giant Platelet Syndromes

In addition to BSS, there are several other inherited *giant platelet syndromes*. See Table 40-1 and Mhawech and Saleem⁴⁰ for a more complete discussion of these syndromes. **Box 41-2** provides a listing of the inherited giant platelet syndromes.

Giant Platelets with Velocardiofacial Syndrome

This disorder is considered an autosomal recessive heterozygous variant of BSS. It is characterized by mild thrombocytopenia (with platelet counts ranging from 100 to 220 $\times 10^9/L$), giant platelets, and association with velocardiofacial syndrome (VCFS). The clinical picture of VCFS includes velopharyngeal insufficiency, conotruncal heart disease, and learning disabilities. VCFS is considered to be a milder form of DiGeorge syndrome (thymic hypoplasia, conotruncal cardiac defects, and cardiac abnormalities). No bleeding symptoms

BOX 41-2 Inherited Giant Platelet Disorders

- Bernard-Soulier syndrome
- Giant platelets with velocardiofacial syndrome
- Giant platelets with abnormal surface glycoproteins and mitral valve insufficiency
- Familial macrothrombocytopenia with GP IV abnormality
- Montreal platelet syndrome
- Gray platelet syndrome
- May-Hegglin anomaly
- Fechtner syndrome
- Sebastian syndrome
- Hereditary macrothrombocytopenia
- Epstein syndrome
- Mediterranean macrothrombocytopenia

have been reported. Other than increased platelet size, no ultrastructural abnormalities have been observed. GP Ib β is mapped on chromosome 22q11.2,14, which is located in the same region that is deleted in most patients with VCFS. Therefore, patients with a 22q11 deletion are obligate heterozygous carriers for the GP Ib β deletion and are heterozygotes for BSS.

Giant Platelets with Abnormal Surface Glycoproteins and Mitral Valve Insufficiency

This extremely rare autosomal recessive disorder is characterized by thrombocytopenia with platelet counts ranging from 50 to 60 $\times 10^9/L$, giant platelets, and mild bleeding symptoms in association with mitral valve insufficiency. Platelets generally are larger than 20 μm . Other than increased platelet size, ultrastructural characteristics are normal. These patients usually have a mild bleeding disorder expressed primarily as ecchymosis and epistaxis occurring early in childhood. This disorder is distinguished from other inherited giant platelet syndromes by mitral valve insufficiency. Platelet surface glycoproteins GP Ia, GP Ic, and GP IIa are absent, while GP Ib, GP IIb, and GP IIIa are normal. The surface glycoproteins absent in this disorder do not seem to have a clear role. However, the abnormal bleeding tendency clearly reflects a platelet dysfunction that could result from a defect in glycoprotein composition, the absence or defective function of an anchor protein necessary to attach the glycoproteins to the cytoskeleton, or both. The cause of large platelets is unclear.

Familial Macrothrombocytopenia with GP IV Abnormality

Familial macrothrombocytopenia with GP IV abnormality is an extremely rare, autosomal dominant disorder characterized by thrombocytopenia, giant platelets, and variable degrees of bleeding tendencies. The thrombocytopenia varies, and the platelet count ranges from 45 $\times 10^9/L$ to normal. GP IV is present in normal concentration, but there seems to be a defect in its glycosylation. GP IV seems to be involved in the early stages of adhesion, and abnormal function may be related to the mild bleeding tendency. Peripheral blood smears show large platelets and neutrophils without inclusions. At this point, the cause of large platelets remains to be clarified.

Montreal Platelet Syndrome

Montreal platelet syndrome (MPS) is characterized by severe thrombocytopenia, giant platelets, spontaneous platelet aggregation, and bleeding symptoms, including significant bruising and episodes of hemorrhage. Of the inherited giant platelet syndromes, MPS patients have the most severe thrombocytopenia with platelet counts ranging from 5 to 40 $\times 10^9/L$. Ultrastructurally, the platelets are large with no other abnormalities. Glycoprotein analysis is normal. It has been suggested that calpain, which is known to be involved in the cleavage of cytoskeleton proteins—in particular, actin-binding protein and talin—may have a role in spontaneous platelet aggregation. Low calpain proteolytic activity in MPS platelets has been

demonstrated and may result in defective regulation of binding sites of platelets for adhesive proteins. As a result, platelet binding sites may be abnormally exposed, leading to an abnormal binding of adhesive proteins to the exposed platelet surfaces and spontaneous aggregation. The pathogenesis of giant platelets, severe thrombocytopenia, spontaneous aggregation, and the role of calpain remain to be clarified.

Gray Platelet Syndrome

See α -Granule Deficiency: Gray Platelet Syndrome later in this chapter for a discussion of this syndrome.

May-Hegglin Anomaly

May-Hegglin anomaly (MHA) is characterized by the presence of giant platelets, thrombocytopenia with platelet counts 60 to 100 $\times 10^9/L$, Döhle body-like neutrophil inclusions, and mild bleeding symptoms (Figure 29-5). MHA is the most common of the inherited giant platelet disorders and is inherited as an autosomal dominant trait. There appear to be two populations of platelets in MHA. Normal-sized platelets have normal ultrastructure and function. The large platelets show clear evidence of abnormal distribution of the platelet microtubule system, and this may be responsible for the large size and defective platelet function that could lead to bleeding symptoms. The cause of the thrombocytopenia is unknown. Chapter 40 provides a detailed discussion of this disorder.

Fechtner Syndrome

Fechtner syndrome is characterized by thrombocytopenia with platelet counts ranging from 30 to 90 $\times 10^9/L$, giant platelets, and the development of deafness, cataracts, and nephritis. Its mode of inheritance appears to be autosomal dominant. Glaucoma and cataracts occur at an early age. Nephritis progresses to end-stage renal failure by the age of 20 to 40 years, necessitating hemodialysis and renal transplant. By the third decade of life, there is usually high-frequency hearing loss. Neutrophils and occasionally eosinophils contain one or more 1- to 2- μm irregularly shaped cytoplasmic inclusions that appear pale blue with Wright-Giemsa stain. They are smaller and less intensely stained than the inclusions found in May-Hegglin anomaly. The thrombocytopenia in this disorder may be due to ineffective megakaryocytopoiesis and thrombopoiesis as reflected by large numbers of megakaryocytes, abnormal morphology, and low platelet count.

Sebastian Syndrome

Sebastian syndrome is an extremely rare autosomal dominant thrombocytopenia characterized by giant platelets, neutrophil inclusions, a mild bleeding disorder, and no other clinical manifestations. The thrombocytopenia is mild with platelet counts ranging from 40 to 120 $\times 10^9/L$. Mild bleeding such as epistaxis may occur in early childhood, but severe postoperative hemorrhage has been observed. Peripheral blood smears show large platelets and faintly blue cytoplasmic inclusions in the neutrophils, similar in appearance to Döhle bodies. At the ultrastructural level, the platelets are enlarged but have normal structural elements. The pathogenesis

of the thrombocytopenia and the neutrophil inclusions remains to be clarified.

Hereditary Macrothrombocytopenia

Hereditary macrothrombocytopenia is a rare disorder characterized by mild thrombocytopenia with platelet counts ranging from 50 to $123 \times 10^9/L$, giant platelets, bleeding tendency, and high-frequency hearing loss. It appears to be inherited in an autosomal dominant fashion. These patients have a mild bleeding tendency, the most frequent symptoms being gingival bleeding after brushing teeth, epistaxis, easy bruising, and menorrhagia. Bleeding symptoms appear early in childhood, but hearing loss appears later. Ultrastructurally, the platelets are large but have no additional abnormalities. Interestingly, flow cytometry studies have shown a distinct population of platelets in these patients that have glycoprotein A on their surface. Glycoprotein A is generally considered an erythroid-specific protein and is not expressed on normal platelets. However, the pathogenesis of thrombocytopenia and giant platelets remains unclear, as does the presence of glycoprotein A on the surface of the giant platelets in this disorder.

Epstein Syndrome

Epstein syndrome is a very rare, autosomal dominant disorder characterized by thrombocytopenia, large platelets, and mild bleeding diathesis, in association with nephritis and high-frequency hearing loss. These patients also have persistent proteinuria, microscopic hematuria, and moderate hypertension. The mild bleeding tendency includes epistaxis, gastrointestinal bleeding, and female genital tract bleeding. Bleeding symptoms occur early in life but may disappear later. The thrombocytopenia is often severe, with platelet counts ranging from 30 to $60 \times 10^9/L$. The peripheral blood smear shows increased platelet size in approximately 50% of the platelets. Ultrastructurally, the giant platelets are spherical and have a prominent surface-connected open canalicular system leading to a sponge-like cytoplasm. The pathogenesis of the thrombocytopenia and large platelets has not been determined.

Mediterranean Macrothrombocytopenia

Mediterranean macrothrombocytopenia is characterized by thrombocytopenia and large platelets. There is no bleeding or other symptom. This disorder has a high prevalence among persons originating from Greece, Italy, and the Balkan Peninsula. The incidence is unknown. The thrombocytopenia is mild, with platelet counts ranging from 89 to $290 \times 10^9/L$. The peripheral blood smear shows large platelets, and electron microscopy reveals no other abnormalities. Of unknown significance, stomatocytes are also present on peripheral blood smears. The pathogenesis of this disorder remains to be elucidated.

Disorders of Platelet Secretion (Release Reactions)

Of the hereditary platelet function defects, disorders involving storage pool defects and the release reaction are the most common. The clinical features of this group of disorders are mucocutaneous hemorrhage and hematuria, epistaxis, and easy and

spontaneous bruising. Petechiae are less common than in other qualitative platelet disorders. Hemorrhage is rarely severe but may be exacerbated by ingestion of aspirin or other antiplatelet agents. In most of these disorders, the platelet count is normal, and the bleeding time is usually, although not always, prolonged. Platelet aggregation abnormalities are usually seen but vary, depending on the disorder.^{2,8,41,42}

Storage Pool Diseases

Platelet disorders of the storage pool type can be related to defects of the dense granules or defects of the α -granules. Box 41-3 lists these disorders.

Dense Granule Deficiencies. The inheritance of *dense granule deficiency* does not follow a single mode, and it is likely that a variety of genetic abnormalities lead to the development of this disorder. Dense granule deficiencies can be subdivided into deficiency states associated with albinism and those in otherwise normal individuals (nonalbinos).

In the platelets of nonalbinos, there is evidence for the presence of dense granule membranes in normal to near-normal numbers, which suggests that the disorder arises from an inability to package the dense granule contents.^{43,44} Serotonin accumulates in normal dense granules by an active uptake mechanism in which plasma serotonin is transported by a specific carrier-mediated system across the plasma membrane into the cytoplasm, and a second carrier-mediated system in the dense granule membrane transports serotonin from the cytoplasm to the interior of the dense granules.⁴⁵ These transport mechanisms are used in the serotonin release assay employed to detect heparin-dependent antiplatelet antibodies (Chapter 40). In addition to serotonin transport mechanisms, a nucleotide transporter MRP4 (*ABCC4*) that is highly expressed in platelets and dense granules has been identified. It would be expected that mutations in the gene for this transporter could affect nucleotide accumulation in dense granules.⁴⁶

As an isolated abnormality, dense granule deficiency does not typically result in a serious hemorrhagic problem. Bleeding is usually mild and most often is limited to easy bruisability.

Dense granule deficiency affects the results of platelet aggregation tests. Dense granules are intracellular storage sites for ADP, adenosine triphosphate, calcium, pyrophosphate, and serotonin. The contents of these granules are extruded when platelet secretion is induced, and secreted ADP plays a major

BOX 41-3 Platelet Storage Pool Diseases

Dense granule deficiencies

Hermansky-Pudlak syndrome

Chédiak-Higashi syndrome

Wiskott-Aldrich syndrome

Thrombocytopenia-absent radius (TAR) syndrome

α -Granule deficiencies

Gray platelet syndrome

role in the propagation of platelet activation, recruitment, and aggregation and growth of the hemostatic plug.

In patients with dense granule deficiency, addition of arachidonic acid to platelet-rich plasma fails to induce an aggregation response (Chapter 42). Epinephrine and low-dose ADP induce a primary wave of aggregation, but a secondary wave is missing. Responses to low concentrations of collagen are decreased to absent, but a high concentration of collagen may induce a near-normal aggregation response.^{42,45} This aggregation pattern is caused by the lack of ADP secretion and is almost identical to the pattern observed in patients taking aspirin.

Hermansky-Pudlak Syndrome. In addition to occurring as an isolated problem, dense granule deficiency is found in association with several disorders. *Hermansky-Pudlak syndrome* is an autosomal recessive disorder characterized by tyrosinase-positive oculocutaneous albinism, defective lysosomal function in a variety of cell types, ceroid-like deposition in the cells of the reticuloendothelial system, and a profound platelet dense granule deficiency.⁴⁷ Several of the mutations responsible for Hermansky-Pudlak syndrome have been mapped to chromosome 19. Mutations in at least seven genes individually can give rise to Hermansky-Pudlak syndrome. These genes encode for proteins that are involved in intracellular vesicular trafficking and are active in the biogenesis of organelles.⁴⁸ Whereas the bleeding associated with most dense granule deficiencies is rarely severe, Hermansky-Pudlak syndrome seems to be an exception. Most bleeding episodes in Hermansky-Pudlak syndrome are not severe; however, lethal hemorrhage has been reported, and in one series hemorrhage accounted for 16% of deaths in patients with Hermansky-Pudlak syndrome. A unique morphologic abnormality has been described in the platelets of four families with Hermansky-Pudlak syndrome. This abnormality consists of marked dilation and tortuosity of the surface-connecting tubular system (the so-called Swiss cheese platelet).^{2,10,20,49}

Chédiak-Higashi Syndrome. This is a rare autosomal recessive disorder characterized by partial oculocutaneous albinism, frequent pyogenic bacterial infections, giant lysosomal granules in cells of hematologic (Figure 29-4) and nonhematologic origin, platelet dense granule deficiency, and hemorrhage.

The *Chédiak-Higashi syndrome* protein gene is located on chromosome 13, and a series of nonsense and frameshift mutations all result in a truncated Chédiak-Higashi syndrome protein that gives rise to a disorder of generalized cellular dysfunction involving fusion of cytoplasmic granules.

The disorder progresses to an accelerated phase in 85% of patients with Chédiak-Higashi syndrome and is marked by lymphocytic proliferation in the liver, spleen, and marrow and macrophage accumulation in tissues. During this stage, the pancytopenia worsens, which leads to hemorrhage and ever-increasing susceptibility to infection; the result is death at an early age. Initially, bleeding is increased because of dense granule deficiency and consequent defective platelet function. During the accelerated phase, however, the thrombocytopenia also contributes to a prolonged bleeding tendency. Bleeding

episodes vary from mild to moderate but worsen as the platelet count decreases.^{10,20}

Wiskott-Aldrich Syndrome. This is a rare X-linked disease caused by mutations in the gene that encodes for a 502-amino acid protein—the *Wiskott-Aldrich syndrome* protein (WASp)—that is found exclusively in hematopoietic cells, including lymphocytes. WASp plays a crucial role in actin cytoskeleton remodeling. T cell function is defective due to abnormal cytoskeletal reorganization, leading to impaired migration, adhesion, and insufficient interaction with other cells. There is a wide range of disease severity associated with Wiskott-Aldrich syndrome (WAS) gene mutations that range from the classic form of WAS with autoimmunity and/or malignancy to a milder form with isolated microthrombocytopenia (X-linked thrombocytopenia; XLT) (Chapter 40) to X-linked neutropenia (XLN). Approximately 50% of patients with WAS gene mutations have the WAS phenotype, and the other half have the XLT phenotype. WAS gene mutations causing XLN are very rare.⁵⁰ Homozygous mutations of the WIPF1 gene on chromosome 2 that encodes WASp-interacting protein (WIP)—a cytoplasmic protein required to stabilize WASp—can also cause a WAS phenotype.⁵¹

The classic form of WAS is characterized by susceptibility to infections associated with immune dysfunction with recurrent bacterial, viral, and fungal infections, microthrombocytopenia, and severe eczema. Thrombocytopenia is present at birth, but the full expression of WAS develops over the first 2 years of life. Individuals with this disorder lack the ability to make antipolysaccharide antibodies, which results in a propensity for pneumococcal sepsis. Patients with classic WAS tend to develop autoimmune disorders, lymphoma, or other malignancies, often leading to early death. Bleeding episodes are typically moderate to severe. In WAS, a combination of ineffective thrombocytopoiesis and increased platelet sequestration and destruction accounts for the thrombocytopenia. As with all X-linked recessive disorders, it is found primarily in males.^{9,10,20,33,52}

Wiskott-Aldrich platelets are also structurally abnormal. The number of dense granules is decreased, and the platelets are small, a feature of diagnostic importance. Other than in WAS, such small platelets are seen only in TORCH (*Toxoplasma*, other agents, rubella virus, cytomegalovirus, herpesvirus) infections. Diminished levels of stored adenine nucleotides are reflected in the lack of dense granules observed on transmission electron micrographs. The platelet aggregation pattern in WAS is typical of a storage pool deficiency. The platelets show a decreased aggregation response to ADP, collagen, and epinephrine and lack a secondary wave of aggregation in response to these agonists (Chapter 42). The response to thrombin is normal, however.^{10,20} The most effective treatment for the thrombocytopenia seems to be splenectomy, which would be consistent with peripheral destruction of platelets. Platelet transfusions may be needed to treat hemorrhagic episodes. Bone marrow transplantation also has been attempted with some success.^{20,53}

Thrombocytopenia with absent radii syndrome. TAR (Chapter 40) is a rare autosomal recessive disorder characterized

by the congenital absence of the radial bones (the most pronounced skeletal abnormality), numerous cardiac and other skeletal abnormalities, and thrombocytopenia (90% of cases). It is mentioned here because the platelets have structural defects in dense granules with corresponding abnormal aggregation responses. Marrow megakaryocytes may be decreased in number, immature, or normal.^{10,54}

α -Granule Deficiency: Gray Platelet Syndrome. The α -granules are the storage site for proteins (Chapter 13) produced by the megakaryocyte (e.g., platelet-derived growth factor, thrombospondin, and platelet factor 4) or present in plasma and taken up by platelets and transported to α -granules for storage (e.g., albumin, immunoglobulin G [IgG], and fibrinogen). There are 50 to 80 α -granules per platelet, which are primarily responsible for the granular appearance of platelets on stained blood films.

Gray platelet syndrome, a rare disorder first described in 1971, is characterized by the specific absence of morphologically recognizable α -granules in platelets. The disorder is inherited in an autosomal recessive fashion. Clinically, gray platelet syndrome is characterized by lifelong mild bleeding tendencies, moderate thrombocytopenia, fibrosis of the marrow, and large platelets whose gray appearance on a Wright-stained blood film is the source of the name of this disorder.^{2,8,10,55} More recently, a mutation in region 3p21 involving the gene NBEAL2 has been identified. This gene is crucial in the development of α -granules.^{56,57}

In electron photomicrographs of platelets and megakaryocytes, the platelets appear to have virtually no α -granules, although they do contain vacuoles and small α -granule precursors that stain positive for VWF and fibrinogen. Other types of granules are present in normal numbers. The membranes of the vacuoles and the α -granule precursors have P-selectin (CD62) and GP IIb/IIIa, and these proteins can be translocated to the cell membrane on stimulation with thrombin. This indicates that these structures are α -granules that cannot store the typical α -granule proteins. This may provide an explanation for the observation that, in gray platelet syndrome, the plasma levels of platelet factor 4 and β -thromboglobulin are increased because while the proteins normally contained in α -granules are produced, storage in those granules is not possible. As a result, they are released into the circulation. Most patients develop early-onset myelofibrosis, which can be attributed to the inability of megakaryocytes to store newly synthesized platelet-derived growth factors.⁴⁴

Treatment of severe bleeding episodes may require platelet transfusions. Few other treatments are available for these patients. Cryoprecipitate has been used to control bleeding. Desmopressin acetate was found to shorten the bleeding time and has been used as successful prophylaxis in a dental extraction procedure. Some authors believe that desmopressin acetate should be the initial therapy of choice.^{2,8,44,58,59}

Other Storage Pool Diseases. A rare disorder in which both α -granules and dense granules are deficient is known as *α -dense storage pool deficiency*. It seems to be inherited as an

autosomal dominant characteristic. In these patients, other membrane abnormalities also have been described.³³

Quebec platelet disorder is an autosomal dominant bleeding disorder that results from a deficiency of multimerin (a multimeric protein that is stored complexed with factor V in α -granules) and shows protease-related degradation of many α -granule proteins, even though α -granule structure is maintained. Thrombocytopenia may be present, although it is not a consistent feature.³³

Thromboxane Pathway Disorders: Aspirin-Like Effects

Platelet secretion requires the activation of several biochemical pathways. One such pathway is the one leading to thromboxane formation. A series of phospholipases catalyze the release of arachidonic acid and several other compounds from membrane phospholipids. Arachidonic acid is converted to intermediate prostaglandins by cyclooxygenase and to thromboxane A_2 by thromboxane synthase (Figure 13-20). Thromboxane A_2 and other substances generated during platelet activation cause mobilization of ionic calcium from internal stores into the cytoplasm, occupancy of several activation receptors, and initiation of a cascade of events resulting in secretion and aggregation of platelets (Figure 13-21).⁶⁰

Several acquired or congenital disorders of platelet secretion can be traced to structural and functional modifications of arachidonic acid pathway enzymes. Inhibition of cyclooxygenase occurs on ingestion of drugs such as aspirin and ibuprofen. As a result, the amount of thromboxane A_2 produced from arachidonic acid depends on the degree of inhibition. Thromboxane A_2 is required for storage granule secretion and maximal platelet aggregation in response to epinephrine, ADP, and low concentrations of collagen.^{6,10,20,61}

Hereditary absence or abnormalities of the components of the thromboxane pathway are usually termed *aspirin-like defects* because the clinical and laboratory manifestations resemble those that follow aspirin ingestion. Platelet aggregation responses are similar to those in dense granule storage pool disorders (see earlier). Unlike in storage pool disorders, however, ultrastructure and granular contents are normal. Deficiencies of the enzymes cyclooxygenase and thromboxane synthase are well documented, and dysfunction or deficiency of thromboxane receptors is known.⁴⁴

Inherited Disorders of Other Receptors and Signaling Pathways

Collagen Receptors

The $\alpha_2\beta_1$ (GP Ia/IIa) integrin is one of the collagen receptors in the platelet membrane. A deficiency of this receptor has been reported in a patient who lacked an aggregation response to collagen, whose platelets did not adhere to collagen, and who had a lifelong mild bleeding disorder.⁶² A deficiency in another collagen receptor, GP VI, also has been reported in patients with mild bleeding. The platelets of these patients failed to aggregate in response to collagen, and adhesion to collagen also was impaired.⁶³ A family with gray platelet syndrome and defective collagen adhesion has been described.

Affected members of the family have a severe deficiency of GP VI.⁶⁴

ADP Receptors

Platelets seem to contain at least three receptors for ADP. P2X₁ is linked to an ion channel that facilitates calcium ion influx. P2Y₁ and P2Y₁₂ (P2T_{AC}) are members of the seven-transmembrane domain (STD) family of G protein-linked receptors (Chapter 13). P2Y₁ is thought to mediate calcium mobilization and shape change in response to ADP. Pathology of the P2Y₁ receptor has not yet been reported. P2Y₁₂ is thought to be responsible for macroscopic platelet aggregation and is coupled to adenylate cyclase through a G-inhibitory (G_i) protein complex.⁶⁵ Some patients have been reported to have decreased platelet aggregation in response to ADP but normal platelet shape change and calcium mobilization. These patients have an inherited deficiency of the P2Y₁₂ receptor.⁶⁶⁻⁶⁸ Bleeding problems seem to be relatively mild in these patients, but the only treatment for severe bleeding is platelet transfusion.

Epinephrine Receptors

Congenital defects of the α_2 -adrenergic (epinephrine) receptor associated with decreased platelet activation and aggregation in response to epinephrine are known. The receptors that mediate aggregation in response to epinephrine, ADP, and collagen are STD receptors, as are the protease-activated receptors (PARs) for thrombin. So far, defects in the PAR receptors have not been described.³³

Calcium Mobilization Defects

A group of intracellular defects that affect platelet function includes defects in which all elements of the thromboxane pathway are normal, but insufficient calcium is released from the dense tubular system, and the cytoplasmic concentration of ionic calcium in the cytoplasm never reaches levels high enough to support secretion. This group of disorders is often referred to as *calcium mobilization defects*. These represent a heterogeneous group of disorders in which the defects reside in the various intracellular signaling pathways, including defects in G protein subunits and phospholipase C isoenzymes.^{20,69,70}

Scott Syndrome

Scott syndrome is a rare autosomal recessive disorder of calcium-induced membrane phospholipid scrambling (necessary for coagulation factor assembly) and thrombin generation on platelets. Platelets secrete and aggregate normally but do not transport phosphatidylserine and phosphatidylethanolamine from the inner leaflet to the outer leaflet of the plasma membrane. This phospholipid “flip” normally occurs during platelet activation and is essential for the binding of vitamin K-dependent clotting factors. In the membrane of resting platelets, phosphatidylserine and phosphatidylethanolamine are restricted to the inner leaflet of the plasma membrane, and phosphatidylcholine is expressed on the outer leaflet. This asymmetry is maintained by the enzyme aminophospholipid translocase.⁷¹ When platelets are activated, the asymmetry is

lost, and phosphatidylserine and phosphatidylethanolamine flip to the outer leaflet and facilitate the assembly of clotting factor complexes. The phospholipid flip is mediated by a calcium-dependent enzyme, scramblase.⁷² In Scott syndrome, platelet plug formation (including adhesion, aggregation, and secretion) occurs normally, but clotting factor complexes do not assemble on the activated platelet surface, and thrombin generation is absent or much reduced. Because lack of thrombin generation leads to inadequate fibrin, the platelet plug is not stabilized, and a bleeding diathesis results.^{73,74}

Stormorken Syndrome

Lastly, *Stormorken syndrome* is a condition in which platelets are always in an “activated” state and express phosphatidylserine on the outer leaflet of the membrane without prior activation. It has been postulated that patients with this syndrome have a defective aminophospholipid translocase.⁷⁵

Acquired Defects of Platelet Function

Therapeutic drugs have been developed with the target to inhibit platelet function. Other drugs and certain agents have been identified that also affect platelet function. The agents that will be discussed are summarized in Table 41-1.

Drug-Induced Defects

Drugs That Inhibit the Prostaglandin Pathway. Unlike inherited disorders of platelet function, which are rare, acquired disorders of platelet function are commonly encountered. The most frequent cause of acquired platelet dysfunction is drug ingestion, with aspirin and other drugs that inhibit the platelet prostaglandin synthesis pathways being the most common culprits.

TABLE 41-1 Antiplatelet Agents

Drug	Mechanism of Action
Therapeutic Antiplatelet Agents	
Aspirin	Irreversible inhibition of COX1
Naproxen	Reversible inhibition of COX1
Sulfipyrazone	Reversible inhibition of COX1
Ibuprofen (and related)	Reversible inhibition of COX1
Clopidogrel	Irreversible inhibition of P2Y ₁₂ receptors
Prasugrel receptors	Irreversible inhibition of P2Y ₁₂ receptors
Ticagrelor receptors	Reversible inhibition of P2Y ₁₂ receptors
Abciximab	Inhibition of GP IIb/IIIa (α_{IIb}/β_3)
Eptifibatide	Inhibition of GP IIb/IIIa (α_{IIb}/β_3)
Tirofiban	Inhibition of GP IIb/IIIa (α_{IIb}/β_3)
Dipyridamole	Inhibition of PDE (and cAMP breakdown)
Aggrenox	Inhibition of COX1 and PDE
Drugs with Antiplatelet Effects	
Alcohol	Inhibition of thromboxane synthesis (?)
Nitrofurantoin	Unknown
Dextrans	Interference with membrane function
Hydroxyethyl (HETA) starch	Interference with membrane function

A single 200-mg dose of *acetylsalicylic acid* (*aspirin*) can irreversibly acetylate 90% of the platelet cyclooxygenase (Figure 13-20). In platelets the acetylated cyclooxygenase (cyclooxygenase-1, or COX-1) enzyme is completely inactive. Platelets lack a nucleus and cannot synthesize new enzymes. The inhibitory effect is permanent for the circulatory life span of the platelet (7 to 10 days).

Endothelial cells synthesize new cyclooxygenase, and endothelial cell cyclooxygenase seems to be less sensitive to aspirin than the platelet enzyme, at least at low dosages. This has led to the view that low dosages of aspirin may be better than higher dosages for cardiovascular protection, because platelet thromboxane production is inhibited, whereas endothelial cells recover prostacyclin production with its accompanying antiplatelet effects. Others argue that inhibition of platelet function is the more important effect and that higher dosages of aspirin are better for this purpose. For these reasons, there are wide-ranging opinions as to the optimal dosage of aspirin.

What is lost in these arguments is that endothelial cells also produce another potent platelet inhibitor, nitric oxide (NO), and its production is not affected by aspirin. Although aspirin may inhibit a proaggregatory mechanism (thromboxane production) and an antiaggregatory mechanism (prostacyclin production) in endothelial cells, the NO platelet inhibitory mechanism is not affected.

It may be necessary to define a test system to determine the optimal dosage of aspirin for cardiovascular protection on an individual basis because some patients have, or develop, *aspirin resistance*, and a dosage that previously was sufficient to inhibit platelet function effectively may no longer be able to produce that effect. In addition, unlike the practice with almost all other therapeutic agents, a single dose of aspirin is usually prescribed in a "one dose fits all" fashion (e.g., 325 mg) without regard to the patient's weight, age, health status, or other measurable parameters. This practice is based on the assumption that the biologic effect will be the same in all patients. Evidence is emerging, however, that there are considerable interindividual differences in the response to a single dose of aspirin.^{3,20,76-78} One study has shown that patients who do not respond well to aspirin have worse cardiovascular outcomes than patients who respond well.⁷⁹ The VerifyNow (Accumetrics, San Diego, CA) is one system that provides measurement of a patient's response to antiplatelet medication (Chapters 42 and 44). Individual tests for aspirin, P2Y₁₂ receptor inhibitors (clopidogrel, prasugrel, ticagrelor), or GP IIb/IIIa receptor inhibitors (abciximab, eptifibatid, tirofiban) are available on the VerifyNow.

Individuals known to have a defect in their hemostatic mechanism, such as a storage pool deficiency, thrombocytopenia, a vascular disorder, or VWD, may experience a marked increase in bleeding tendency after aspirin ingestion, and such individuals should be advised to avoid the use of aspirin and related agents.²⁰

The list of drugs affecting the prostaglandin pathway that converts arachidonic acid to thromboxane is long and beyond the scope of this chapter. Many of these drugs inhibit cyclooxygenase, but, unlike with aspirin and closely related compounds,

the inhibition is reversible. These drugs are said to be *competitive inhibitors of cyclooxygenase*, and as the blood concentration of the drug decreases, platelet function is recovered. This group of drugs includes *ibuprofen and related compounds*, such as ketoprofen and fenoprofen, naproxen, and sulfinpyrazone. In contrast to aspirin, most of these agents have little effect on the platelet function tests (Chapters 42 and 44). Except for their potential to irritate the gastric mucosa, these drugs have not been reported to cause clinically important bleeding.^{6,11,20,80} Interestingly, ibuprofen appears to have a prothrombotic effect when ingested within 2 hours of aspirin because it blocks the acetylation site for aspirin on COX-1. Patients taking aspirin should be cautioned to avoid ibuprofen and related drugs near the time of aspirin ingestion.

The association of *chronic alcohol consumption* with thrombocytopenia is well known. Chronic, periodic, and even acute alcohol consumption may result in a transient decrease in platelet function, however, and the inhibitory effect seems to be more pronounced when alcohol is consumed in excess. Most patients who are scheduled to undergo a medical procedure in which there may be hemostatic challenge are advised to abstain from alcohol consumption for about 3 days before the procedure. The impaired platelet function seems to be related at least in part to inhibition of thromboxane synthesis. A reduced platelet count and impaired platelet function may contribute to the increased incidence of gastrointestinal hemorrhage associated with chronic excessive alcohol intake.^{6,20,81,82}

Drugs That Inhibit Membrane Function. Many drugs interact with the platelet membrane and cause a clinically significant platelet function defect that may lead to hemorrhage. Some of these drugs are useful antiplatelet agents, whereas for many other drugs, their effects on the platelet membrane are an adverse side effect.⁶⁰

P2Y₁₂ (ADP) Receptor Inhibitors. The *thienopyridine derivatives* clopidogrel, prasugrel, their predecessor ticlopidine, and the nucleoside *ticagrelor* are antiplatelet agents that bind to the P2Y₁₂ platelet receptor thus inhibiting platelet function. These drugs are for treatment of patients with arterial occlusive disease for prevention of myocardial infarction, for patients with cerebrovascular disease for reduction of the risk of thrombotic stroke, for stroke and myocardial infarction prophylaxis, and for patients who are intolerant of aspirin.

In contrast to the effects of aspirin, the effects of these agents do not reach a steady state for 3 to 5 days, although a steady state can be reached sooner with a loading dose. As prophylactic agents, they have been shown to be as efficacious as aspirin. P2Y₁₂ inhibitors and aspirin are often used in combination to prevent arterial thrombosis, primarily based on the synergistic action of these two drugs, which inhibit platelet function by different mechanisms.

The mechanism of action for P2Y₁₂ inhibitors is binding to the platelet membrane STD receptors for ADP and the prevention of ADP binding to those receptors.⁶⁶ As with aspirin inhibition of cyclooxygenase, the effect of the irreversible thienopyridines on platelet recovery of function following drug cessation is 50% of normal at 3 days, and complete at 7 to 10 days.⁸³

The major effect of the binding of these drugs to P2Y₁₂ receptors appears to be inhibition of stimulus-response coupling between those receptors and fibrinogen binding to GP IIb/IIIa. As a consequence, platelet activation and aggregation induced by ADP are markedly inhibited, and responses to other aggregating agents, such as collagen, are reduced.

Clopidogrel (Plavix), a second-generation thienopyridine derivative, is an effective antiplatelet agent for a variety of clinical applications, though its clinical effectiveness varies from patient to patient based on metabolism to the active drug. Clopidogrel is a pro-drug and requires conversion to the active drug by the P450 enzyme systems of the liver. For clopidogrel, the isoform of P450 involved in clopidogrel metabolism is CYP2C19 (a.k.a. *CYP2C19*). There are numerous mutations in *CYP2C19* that result in decreased activity of the enzyme and therefore inhibit the conversion of clopidogrel to the active drug. *CYP2C19**1/*1 (wild type) represents two normal functioning alleles and normal metabolism of clopidogrel to the active drug. Hypofunctional alleles are *CYP2C19**2 to *10, while *CYP2C19**17 is a hyperfunctional allele. Patients with one of the mutations resulting in decreased activity (e.g., *CYP2C19**1/*2) are considered intermediate metabolizers, and the usual dose of clopidogrel does not achieve the degree of platelet inhibition desired. These individuals remain at increased risk for thromboembolic events. Increasing the dose of clopidogrel may increase the degree of platelet inhibition, but it does not decrease the thromboembolic risk. Those who have two hypofunctional alleles (*CYP2C19**2/*2, *2/*3, or any combination of two hypofunctional alleles) are poor metabolizers and do not derive significant benefit from clopidogrel therapy. Approximately 25% of individuals have one or two hypofunctional alleles of *CYP2C19* and are considered to be *clopidogrel resistant*. In contrast, those with a *17 allele are rapid metabolizers and convert clopidogrel to the active drug at a faster rate. This results in increased blood levels of the active drug following a dose of clopidogrel and an increased risk of bleeding. Those with one normal allele and one *17 allele (*1/*17) are considered to be rapid metabolizers and those who are *17/*17 are ultra-rapid metabolizers. Finally, individuals with one hypofunctional and one hyperfunctional allele (e.g., *2/*17) have normal to intermediate clopidogrel metabolism. There are a variety of molecular methods available to test for the most common alleles of *CYP2C19*. Although the FDA has recommended pharmacogenetic testing for these alleles, it is not a common practice.

Clopidogrel has more effect on the platelet function tests than aspirin, although there is little difference in the risk of clinical bleeding.⁸⁴ Clopidogrel can occasionally produce major side effects in some patients, including long-lasting neutropenia, aplastic anemia, thrombocytopenia, gastrointestinal distress, and diarrhea.

Prasugrel (Effient) is a third-generation thienopyridine derivative. It has the same mechanism of action as clopidogrel. It is also a pro-drug, but its metabolism to the active form does not require *CYP2C19*. Instead, it is metabolized to the active drug by several enzymes of the cytochrome P450 system, including *CYP3A4* and *CYP2B6*. Because it is activated by several

enzymes, mutations that result in decreased function of one or more of these enzyme have less impact, and the response is much more uniform than clopidogrel. Pharmacogenetic testing for mutations affecting prasugrel activation is not recommended.

Ticagrelor (Brilinta) is a nucleoside and the newest of the P2Y₁₂ inhibitors. While its antiplatelet effect is similar, it has two important differences from prasugrel and clopidogrel. First, it is not a pro-drug and therefore does not require bioactivation. Because it is rapidly absorbed, its antiplatelet effect is predictable and achieved in a short period of time. In addition, ticagrelor binds to a slightly different site on the P2Y₁₂ (ADP) receptor than clopidogrel or prasugrel. This difference results in reversible binding. Therefore, unlike clopidogrel and prasugrel whose effects are irreversible, platelet function returns quite rapidly with cessation of ticagrelor. However, this short half-life (7 to 9 hours) requires twice-daily dosing and may be a compliance issue for some patients.

GP IIb/IIIa (α_{IIb}/β_3) receptor inhibitors. Another target for antiplatelet agents to reduce cardiovascular thrombotic risk is the platelet membrane GP IIb/IIIa (α_{IIb}/β_3) receptor. Interference with the ability of this receptor to bind fibrinogen inhibits platelet aggregation stimulated in response to all of the usual platelet aggregating agents. Results of platelet function studies on platelets from patients receiving therapeutic doses of these drugs essentially mimic those of a mild form of Glanzmann thrombasthenia.

Two different types of agents are included in this group. The first such agent approved for clinical use in the United States was the Fab fragment of the mouse/human chimeric monoclonal antibody 7E3 (c7E3 Fab; *abciximab* [ReoPro]), which binds to GP IIb/IIIa, prevents the binding of fibrinogen, and prevents platelet aggregation. Numerous studies have shown the efficacy of this drug as an antiplatelet and antithrombotic agent.

The second type of agent in this group targets a GP IIb/IIIa recognition site for an arginine-glycine-aspartic acid (RGD) sequence found in fibrinogen and several adhesive proteins. These agents bind to the RGD recognition site, prevent the binding of fibrinogen, and consequently prevent platelet aggregation. These compounds are relatively easily synthesized. *Tirofiban* (Aggrastat) is a nonpeptide RGD mimetic, and *eptifibatid* (Integrilin) is a cyclic heptapeptide that is also an RGD mimetic. When the receptor site is occupied by the drug, GP IIb/IIIa is no longer able to bind fibrinogen or other adhesive proteins and is no longer functional as the aggregation receptor.

The goal of therapy with these drugs is to induce a controlled thrombasthenia-like state. At present, these agents are primarily used in patients undergoing percutaneous coronary intervention and are administered concurrently with heparin and other antiplatelet agents. The use of these agents is limited by the need to administer them by constant intravenous infusion and by their short half-lives. There have been attempts to make an orally active agent of this type, but none has been approved for use.⁸⁵⁻⁸⁷

Other Therapeutic Drugs That Inhibit Platelet Function. Dipyridamole is an inhibitor of platelet phosphodiesterase, the

enzyme responsible for converting cyclic adenosine monophosphate (cAMP) to AMP. Elevation of cytoplasmic cAMP is inhibitory to platelet function, and inhibition of phosphodiesterase allows the accumulation of cAMP in the cytoplasm (Figure 13-21). Dipyridamole alone does not inhibit platelet aggregation in response to the usual platelet agonists, but it promotes inhibition of agents that stimulate cAMP formation, such as prostacyclin, stable analogues of prostacyclin, and NO. At one time, dipyridamole, alone or in combination with aspirin, was widely used. By the 1990s, interest in dipyridamole had waned. There has been a resurgence of interest in dipyridamole, however, as a combination agent compounded with aspirin (*Aggrenox*).

Miscellaneous Agents That Inhibit Platelet Function.

Well known for their ability to interfere with platelet function are *antibiotics*. Most of the drugs with this effect contain the β -lactam ring and are either a penicillin or a cephalosporin (Figure 41-4). These drugs can inhibit platelet function tests, but this effect is seen only in patients receiving large parenteral doses and is thus only a problem for hospitalized patients. One postulated mechanism for the antiplatelet effect of these drugs is that they associate with the membrane via a lipophilic reaction and block receptor-agonist interactions or stimulus-response coupling between receptors and fibrinogen binding to GP IIb/IIIa. They also may inhibit calcium influx in response to thrombin stimulation, reducing the ability of thrombin to activate platelets. Although these drugs may prolong the bleeding time test and in vitro aggregation responses to certain agonists, their association with a hemostatic defect severe enough to cause clinical hemorrhage is uncertain and is not predicted by the bleeding time test results.^{3,11,61,88}

Nitrofurantoin is an antibiotic that is not related to the β -lactam drugs but may inhibit platelet aggregation when high concentrations are present in the blood. This drug is not known to cause clinical bleeding, however.⁸⁸

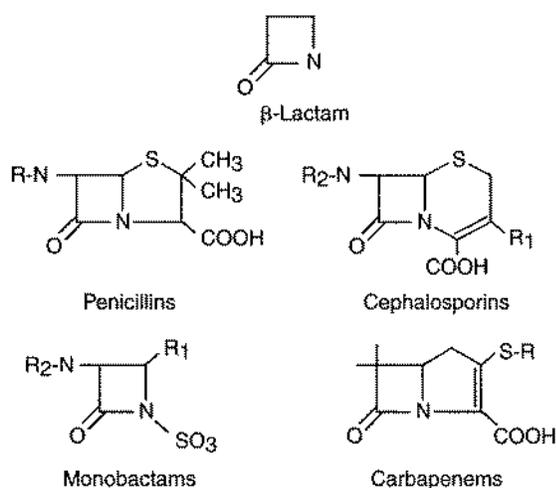


Figure 41-4 Chemical structure of major classes of β -lactam antibiotics. R, Nonspecified side chain. (From Mahon CR, Lehman DE, Manuselis G: *Textbook of diagnostic microbiology*, ed 4, St. Louis, 2011, Saunders.)

The *dextrans*, another class of commonly used drugs, can inhibit platelet aggregation, and impair platelet procoagulant activity when given as an intravenous infusion. These drugs have no effect on platelet function, however, when added directly to platelet-rich plasma. Dextrans are partially hydrolyzed, branched-chain polysaccharides of glucose. The two most commonly used are dextran 70 (molecular mass of 70,000 to 75,000 Daltons) and dextran 40 (molecular mass of 40,000 Daltons), also known as low-molecular-weight dextran. Both drugs are effective plasma expanders and are commonly used for this purpose. Because of their effects on platelets, they have been extensively used as antithrombotic agents. There does not seem to be any increased risk of hemorrhage associated with the use of these agents, but their efficacy in preventing postoperative pulmonary embolism is equal to that of low-dose subcutaneous heparin.^{6,52,81,88}

Hydroxyethyl starch, or hetastarch, is a synthetic glucose polymer with a mean molecular mass of 450,000 Daltons that also is used as a plasma expander. It has effects similar to those of the dextrans. The mechanism of action of these drugs has not been clearly elucidated but is presumed to involve interaction with the platelet membrane.^{6,52,81}

Several other agents of diverse chemical structure and function are known to inhibit platelet function. The mechanisms by which they induce platelet dysfunction are largely unknown. Nitroglycerin, nitroprusside, propranolol, and isosorbide dinitrate are drugs used to regulate cardiovascular function that seem to be able to cause a decrease in platelet secretion and aggregation. Patients taking phenothiazine or tricyclic antidepressants may have decreased secretion and aggregation responses, but these effects are not associated with an increased risk for hemorrhage. Local and general anesthetics may impair in vitro aggregation responses. The same is true of antihistamines. Finally, some radiographic contrast agents are known to inhibit platelet function.⁸⁸

Disorders That Affect Platelet Function

Myeloproliferative Neoplasms. Chronic myeloproliferative neoplasms (MPNs) include *polycythemia vera*, *chronic myelogenous leukemia*, *essential thrombocythemia*, and *myelofibrosis with myeloid metaplasia* (Chapter 33). Platelet dysfunction is a common finding in patients with these disorders. Hemorrhagic complications occur in about one third, thrombosis occurs in another third, and, although it is uncommon, both develop in some patients. These complications are serious causes of morbidity and mortality.

Although the occurrence of hemorrhage or thrombosis in MPN patients is largely unpredictable, certain patterns have emerged. Hemorrhage and thrombosis are less common in chronic myelogenous leukemia than in the other MPNs. Bleeding seems to be more common in myelofibrosis with myeloid metaplasia, but thrombosis is more common in the other MPNs.

Abnormal platelet function has been postulated as a contributing cause. This hypothesis is supported by the observation that bleeding is usually mucocutaneous in nature, and thrombosis may be arterial or venous.

In patients with these disorders, thrombosis may occur in unusual sites, including the mesenteric, hepatic, and portal circulations. Patients with essential thrombocythemia may develop digital artery thrombosis and ischemia of the fingers and toes, occlusions of the microvasculature of the heart, and cerebrovascular occlusions that result in neurologic symptoms.⁸⁸

In MPNs, a variety of platelet function defects have been described, but their clinical importance is uncertain. Platelets have been reported to have abnormal shapes, decreased procoagulant activity, and a decreased number of secretory granules. In essential thrombocythemia, platelet survival may be shortened. The bleeding time is prolonged in only a few patients, and hemorrhage can occur in patients with a normal bleeding time. The risk of thrombosis or hemorrhage correlates poorly with the elevation of the platelet count.

The most common abnormalities are decreased aggregation and secretion in response to epinephrine, ADP, and collagen.⁸⁹ Possible causes of the platelet dysfunction include loss of platelet surface membrane α -adrenergic (epinephrine) receptors, impaired release of arachidonic acid from membrane phospholipids in response to stimulation by agonists, impaired oxidation of arachidonic acid by the cyclooxygenase and lipoxygenase pathways, a decrease in the contents of dense granules and α -granules, and loss of a variety of platelet membrane receptors for adhesion and activation. There seems to be no correlation between a given MPN and the type of platelet dysfunction observed, with the exception that most patients with essential thrombocythemia lack an *in vitro* platelet aggregation response to epinephrine. This observation may be helpful in the differential diagnosis.^{6,11,88,90}

Multiple Myeloma and Waldenström Macroglobulinemia. Platelet dysfunction is observed in approximately one third of patients with *IgA myeloma* or *Waldenström macroglobulinemia*, a much smaller percentage of patients with *IgG multiple myeloma*, and only occasionally in patients with *monoclonal gammopathy* of undetermined significance.

Platelet dysfunction results from coating of the platelet membranes by paraprotein and does not depend on the type of paraprotein present. In addition to interacting with platelets, the paraprotein may interfere with fibrin polymerization and the function of other coagulation proteins.

Almost all patients with malignant paraprotein disorders have clinically significant bleeding, but thrombocytopenia is still the most likely cause of bleeding in these patients. Other causes of bleeding include hyperviscosity syndrome, complications of amyloidosis (e.g., acquired factor X deficiency), and, in rare instances, presence of a circulating heparin-like anticoagulant or fibrinolysis.^{9,81,88}

Cardiopulmonary Bypass Surgery. The use of the *cardiopulmonary bypass machine* (CPB; heart-lung machine) during cardiac surgery induces thrombocytopenia and a severe platelet function defect that assumes major importance in bleeding after surgery. The function defect most likely results from platelet activation and fragmentation in the extracorporeal circuit.

Causes of platelet activation include adherence and aggregation of platelets to fibrinogen (adsorbed onto the surfaces of the bypass circuit material), mechanical trauma and shear stresses, use of blood conservation devices and bypass pump-priming solutions during surgery, hypothermia, complement activation, and exposure of platelets to the blood-air interface in bubble oxygenators.

Some degree of platelet degranulation typically is found after cardiac surgery using the cardiopulmonary bypass machine, which indicates that platelet activation and secretion have occurred during the operation. Platelet membrane fragments, or "microparticles," are found consistently in the blood of these surgical patients, providing additional evidence of the severe mechanical stress encountered by platelets during these procedures.

The severity of the platelet function defect closely correlates with the length of time on the bypass machine. After an uncomplicated surgical procedure, normal platelet function returns in about 1 hour, although the platelet count does not return to normal for several days. Thrombocytopenia is caused by hemodilution, accumulation of platelets on the surfaces of the bypass materials, sequestration or removal of damaged platelets by the liver and reticuloendothelial system, and consumption associated with normal hemostatic processes after surgery.^{81,88}

Liver Disease. Moderate to severe *liver disease* is reported to be associated with a variety of hemostatic abnormalities, including reduction in clotting proteins, reduction of proteins in the natural anticoagulant pathways, dysfibrinogenemia, and excessive fibrinolysis (Chapter 38). Mild to moderate thrombocytopenia is seen in approximately one third of patients with chronic liver disease in association with hypersplenism or as a result of alcohol toxicity.^{8,88}

Abnormal platelet function test results seen in patients with chronic liver disease include reduced platelet adhesion, abnormal platelet aggregation (in response to ADP, epinephrine, and thrombin), abnormal platelet factor 3 (phospholipids) availability, and reduced procoagulant activity. An acquired storage pool deficiency also has been suggested. The abnormal platelet function in these patients may respond to infusion of desmopressin acetate. It is unclear, however, whether desmopressin acetate provides a benefit in preventing bleeding in these patients or is simply correcting an abnormal laboratory test result.

In *chronic alcoholic cirrhosis*, the thrombocytopenia and platelet abnormalities may result from the direct toxic effects of alcohol on bone marrow megakaryocytes. The severe bleeding diathesis associated with end-stage liver disease has many causes, such as markedly decreased or negligible coagulation factor production, excessive fibrinolysis, dysfibrinogenemia, thrombocytopenia, and (occasionally) disseminated intravascular coagulation. Upper gastrointestinal tract bleeding is a relatively common feature of cirrhosis, particularly alcoholic cirrhosis, and recombinant factor VIIa (rVIIa, NovoSeven, Novo Nordisk Inc, Princeton, NJ) has been shown to be effective treatment in some patients.⁹¹

Uremia. This is commonly accompanied by bleeding caused by platelet dysfunction. In *uremia*, guanidinosuccinic acid (GSA) is present in the circulation in higher than normal levels as a result of inhibition of the urea cycle. GSA is dialyzable, and dialysis (peritoneal dialysis or hemodialysis) is usually effective in correcting the prolonged bleeding time test and the abnormal platelet function characteristic of uremia. NO diffuses into platelets; activates soluble guanylate cyclase; and inhibits platelet adhesion, activation, and aggregation.⁹² Because GSA is an NO donor, NO is present in the circulation at higher than normal levels in uremia. Abnormal platelet function is far more common than clinically significant bleeding in uremic patients.^{2,60,62}

Platelet aggregation pattern abnormalities are not uniform, and any combination of defects may be seen. There is evidence of a deficient release reaction, such as lack of primary ADP-induced aggregation, and subnormal platelet procoagulant activity. The bleeding time test is characteristically prolonged in uremia and seems to correlate with the severity of renal failure in these patients. There does not seem to be any significant correlation, however, between the bleeding time test and the risk of clinically significant bleeding. Anemia is an independent cause of prolonged bleeding time, and the severity of anemia in uremic patients correlates with the severity of renal failure. Many uremic patients are treated with recombinant erythropoietin to increase their hematocrit. Maintenance of the hematocrit at greater than 30% also may help to normalize the bleeding times.^{2,81,84}

Bleeding is uncommon in uremic patients and is seen more often with concurrent use of drugs that interfere with platelet function or in association with heparin use in hemodialysis. Platelet concentrates often are used to treat severe hemorrhagic episodes in patients with uremia but usually do not correct the bleeding. Other therapies that are sometimes effective include cryoprecipitate, desmopressin acetate, and conjugated estrogen.^{2,81,84}

Hereditary Afibrinogenemia. Hereditary afibrinogenemia has been documented in more than 150 families. Although it is not truly a platelet function disorder, platelets do not exhibit normal function in the absence or near-absence of fibrinogen. In most patients, the bleeding time is prolonged, and because fibrinogen is essential for normal platelet aggregation, platelet aggregation test results are abnormal. Abnormal results on platelet retention and adhesion studies involving the use of glass beads also have been documented. In addition, results of all clot-based tests (including partial thromboplastin time, prothrombin time, reptilase time, thrombin time, and whole-blood clotting time) are abnormal. Addition of fibrinogen to samples or infusion of fibrinogen into the patient results in correction of the abnormal test results.^{2,93}

A high incidence of hemorrhagic manifestations is found in patients with afibrinogenemia (or severe hypofibrinogenemia). Bleeding is the cause of death in about one third of such patients. Cryoprecipitate or fibrinogen concentrates can be used to treat bleeding episodes. Some patients develop

antibodies to fibrinogen, and this treatment then becomes ineffective.⁹³

Hyperaggregable Platelets

Patients with a variety of disorders associated with thrombosis or increased risk for thrombosis, including hyperlipidemia, diabetes mellitus, peripheral arterial occlusive disease, acute arterial occlusion, myocardial infarction, and stroke, have been reported to have increased platelet reactivity. Platelets from these patients tend to aggregate at lower concentrations of aggregating agents than do platelets from individuals without these conditions.

Sticky platelet syndrome is an inherited disorder with autosomal dominant characteristics and is associated with venous and arterial thromboembolic events. The disorder is characterized by hyperaggregable platelets in response to ADP, epinephrine, or both. In these patients, venous and/or arterial thrombotic events are often associated with emotional stress. Prophylactic treatment of these patients with low-dose aspirin reverses clinical symptoms and normalizes hyperaggregable responses to aggregating agents in the laboratory.

Spontaneous aggregation (aggregation in response to in vitro stirring only) is also an indicator of abnormally increased platelet reactivity and often accompanies increased sensitivity to platelet agonists. The presence of spontaneous aggregation by itself is considered to be consistent with the presence of a hyperaggregable state. Because participation of platelets is necessary for the development of arterial thrombosis, the presence of hyperaggregable platelets is often an indication that an antiplatelet agent should be used as part of a therapeutic or prophylactic regimen for arterial thrombosis.^{81,94,95}

Acquired platelet function defects are seen occasionally in patients with autoimmune disorders, including systemic lupus erythematosus, rheumatoid arthritis, scleroderma, and the immune thrombocytopenias, such as immune thrombocytopenic purpura.⁸⁸

Purified fibrin degradation products can induce platelet dysfunction in vitro. The pathophysiologic relevance of this observation is uncertain because the concentrations of fibrin degradation products required are unlikely to be reached in vivo. Patients with disseminated intravascular coagulation may have reduced platelet function, however, as a result of in vivo stimulation by thrombin and other agonists resulting in in vivo release of granule contents. This has been called *acquired storage pool disease*; the term *exhausted platelets* may be more appropriate.⁹⁶

VASCULAR DISORDERS

The pathophysiology of disorders of vessels and their supporting tissues is obscure. Laboratory studies of platelets and blood coagulation usually yield normal results. The diagnosis is often based on medical history and is made by ruling out other sources of bleeding disorders. The usual clinical sign is the tendency to bruise easily or to bleed spontaneously, especially from mucosal surfaces. Vascular disorders are summarized in [Box 41-4](#).

BOX 41-4 Vascular Disorders³**Hereditary Vascular Disorders**

Hereditary hemorrhagic telangiectasia (Rendu-Osler-Weber syndrome)

Hemangioma-thrombocytopenia syndrome (Kasabach-Merritt syndrome)

Ehlers-Danlos syndrome and other genetic disorders

Acquired Vascular Disorders

Allergic purpura (Henoch-Schönlein purpura)

Paraproteinemia and amyloidosis

Senile purpura

Drug-induced vascular purpuras

Vitamin C deficiency (scurvy)

Purpuras of Unknown Origin

Purpura simplex (easy bruisability)

Psychogenic purpura

Hereditary Vascular Disorders**Hereditary Hemorrhagic Telangiectasia (Rendu-Osler-Weber Syndrome)**

The mode of inheritance of *hereditary hemorrhagic telangiectasia* is autosomal dominant. The vascular defect of this disorder is characterized by thin-walled blood vessels with a discontinuous endothelium, inadequate smooth muscle, and inadequate or missing elastin in the surrounding stroma. Telangiectasias (dilated superficial blood vessels that create small, focal red lesions) occur throughout the body but are most obvious on the face, lips, tongue, conjunctiva, nasal mucosa, fingers, toes, and trunk and under the tongue. The lesions blanch when pressure is applied. The disorder usually becomes manifest by puberty and progresses throughout life. Telangiectasias are fragile and prone to rupture. Epistaxis is an almost universal finding, and symptoms almost always worsen with age. The age at which nosebleeds begin is a good gauge of the severity of the disorder. Although the oral cavity, gastrointestinal tract, and urogenital tract are common sites of bleeding, bleeding can occur in virtually every organ.⁹⁷

The diagnosis of hereditary hemorrhagic telangiectasia is based on the characteristic skin or mucous membrane lesions, a history of repeated hemorrhage, and a family history of a similar disorder.

Patients with hereditary hemorrhagic telangiectasia do well despite the lack of specific therapy and the seriousness of their hemorrhagic manifestations.^{2,10,97} There are several other disorders and conditions in which telangiectasias are present, including cherry-red hemangiomas (common in older men and women), ataxia-telangiectasia (Louis-Bar syndrome), and chronic actinic telangiectasia; they also are seen in association with chronic liver disease and pregnancy.⁹⁷

Hemangioma-Thrombocytopenia Syndrome (Kasabach-Merritt Syndrome)

Kasabach and Merritt originally described the association of a giant cavernous hemangioma (vascular tumor), thrombocytopenia,

and a bleeding diathesis. The hemangiomas are visceral or subcutaneous, but rarely both. External hemangiomas may become engorged with blood and resemble hematomas. Other well-recognized features of *Kasabach-Merritt syndrome* include acute or chronic disseminated intravascular coagulation (Chapters 38 and 39) and microangiopathic hemolytic anemia. A hereditary basis for this syndrome has not been established, but the condition is present at birth. Several treatment modalities are available for the angiomas and the associated coagulopathy and range from corticosteroid therapy to surgery.^{10,98}

Ehlers-Danlos Syndrome and Other Genetic Disorders

Ehlers-Danlos syndrome may be transmitted as an autosomal dominant, recessive, or X-linked trait. It is manifested by hyperextensible skin, hypermobile joints, joint laxity, fragile tissues, and a bleeding tendency, primarily subcutaneous hematoma formation. Eleven distinct varieties of the disorder are recognized. The severity of bleeding ranges from easy bruisability to arterial rupture. The disorder generally can be ascribed to defects in collagen production, structure, or cross-linking, with resulting inadequacy of the connective tissues. Platelet abnormalities have been reported in some patients.¹⁰

Other inherited vascular disorders include pseudoxanthoma elasticum and homocystinuria (autosomal recessive disorders), and Marfan syndrome and osteogenesis imperfecta (autosomal dominant disorders). In addition to vascular defects, Marfan syndrome is characterized by skeletal and ocular defects.¹⁰

Acquired Vascular Disorders**Allergic Purpura (Henoch-Schönlein Purpura)**

The term *allergic purpura* or *anaphylactoid purpura* generally is applied to a group of nonthrombocytopenic purpuras characterized by apparently allergic manifestations, including skin rash and edema. Allergic purpura has been associated with certain foods and drugs, cold, insect bites, and vaccinations. The term *Henoch-Schönlein purpura* is applied when the condition is accompanied by transient arthralgia, nephritis, abdominal pain, and purpuric skin lesions, which are frequently confused with the hemorrhagic rash of immune thrombocytopenic purpura.^{2,10,52}

General evidence implicates autoimmune vascular injury, but the pathophysiology of the disorder is unclear. Preliminary evidence indicates that the vasculitis is mediated by immune complexes containing IgA antibodies. It has been suggested that allergic purpura may represent autoimmunity to components of vessel walls.^{2,10}

Henoch-Schönlein purpura is primarily a disease of children, occurring most commonly in children 3 to 7 years of age. It is relatively uncommon among individuals younger than age 2 and older than age 20. Twice as many boys as girls are affected. The onset of the disease is sudden, often following an upper respiratory tract infection. The infectious organism may damage the endothelial lining of blood vessels, which results in vasculitis. Attempts have been made to implicate a specific infectious agent, particularly β -hemolytic streptococcus.^{2,10}

Malaise, headache, fever, and rash may be the presenting symptoms. The delay in the appearance of the skin rash often

poses a difficult problem in differential diagnosis. The skin lesions are urticarial and gradually become pinkish, then red, and finally hemorrhagic. The appearance of the lesions may be very rapid and accompanied by itching. The lesions have been described as “palpable purpura,” in contrast to the perfectly flat lesions of thrombocytopenia and most other forms of vascular purpura. These lesions are most commonly found on the feet, elbows, knees, buttocks, and chest. Ultimately, a brownish-red eruption is seen. Petechiae also may be present.^{2,10}

As the disease progresses, abdominal pain, polyarthralgia, headaches, and renal disease may develop. Renal lesions are present in 60% of patients during the second to third week of the disorder. Proteinuria and hematuria are commonly present.^{2,8,10}

The platelet count is normal. Tests of hemostasis, including the bleeding time, and tests of blood coagulation, usually yield normal results in patients with allergic purpura. Anemia generally is not present unless the hemorrhagic manifestations have been severe. The white blood cell count and the erythrocyte sedimentation rate are usually elevated. The disease must be distinguished from other forms of nonthrombocytopenic purpura. Numerous infectious diseases that may be associated with purpura also must be considered in the differential diagnosis. Drugs or chemicals sometimes may be implicated.^{2,10}

In the pediatric age group, the average duration of the initial episode is about 4 weeks. Relapses are frequent, usually after a period of apparent well-being. Except for patients in whom chronic renal disease develops, the prognosis is usually good. Occasionally, death from renal failure has occurred. Management is directed primarily at symptomatic relief, because there currently is no effective treatment. Corticosteroids sometimes have been helpful in alleviating symptoms. Most patients recover without treatment.^{2,10}

Paraproteinemia and Amyloidosis

Platelet function can be inhibited by *myeloma proteins*. Abnormalities in platelet aggregation, secretion, and procoagulant activity (Chapter 42) correlate with the concentration of the plasma paraprotein and are likely due to coating of the platelet membrane with the paraprotein. Under these conditions, platelet adhesion and activation receptor functions are inhibited, and the paraprotein coating also inhibits assembly of clotting factors on the platelet surface. High concentrations of paraprotein can cause severe hemorrhagic manifestations as a result of a combination of hyperviscosity and platelet dysfunction. About one third of patients with *IgA myeloma* and *Waldenström macroglobulinemia* and approximately 5% of patients with *IgG myeloma* (usually IgG3) exhibit platelet function abnormalities. Finally, the paraprotein may contribute further to bleeding by inhibiting fibrin polymerization. In these patients, there is poor correlation between abnormal results on laboratory tests (e.g., prothrombin time, activated partial thromboplastin time, thrombin time, bleeding time) and evidence of clinical bleeding. Treatment for the bleeding complications of these disorders is primarily reduction in the level of the paraprotein. This can be accomplished quickly, albeit transiently, by plasmapheresis. Longer-term treatment is usually chemotherapy for the underlying plasma cell malignancy.^{88,99}

Amyloid is a fibrous protein consisting of rigid, linear, non-branching, aggregated fibrils approximately 7.5 to 10 nm wide and of indefinite length. Amyloid is deposited extracellularly and may lead to damage of normal tissues. Various proteins can serve as subunits of the fibril, including monoclonal light chains (λ more frequently than κ). Amyloidosis, the deposition of abnormal quantities of amyloid in tissues, may be primary or secondary and localized or systemic. A discussion of the clinical spectrum of amyloidosis is beyond the scope of this chapter. Purpura, hemorrhage, and thrombosis may be a part of the clinical presentation of patients with amyloidosis, however. Thrombosis and hemorrhage have been ascribed to amyloid deposition in the vascular wall and surrounding tissues. Platelet function has been shown to be abnormal in a few cases, and in rare cases patients may have thrombocytopenia. Current treatments for amyloidosis are not effective.¹⁰⁰

Senile Purpura

Senile purpura occurs more commonly in elderly men than in women and is due to a lack of collagen support for small blood vessels and loss of subcutaneous fat and elastic fibers. The incidence increases with advancing age. The dark blotches are flattened, are about 1 to 10 mm in diameter, do not blanch with pressure, and resolve slowly, often leaving a brown stain in the skin (age spots). The lesions are limited mostly to the extensor surfaces of the forearms and backs of the hands and occasionally occur on the face and neck. With the exception of increased capillary fragility, results of laboratory tests are normal, and no other bleeding manifestations are present.^{2,10}

Drug-Induced Vascular Purpuras

Purpura associated with *drug-induced vasculitis* occurs in the presence of functionally adequate platelets. A variety of drugs are known to cause *vascular purpura*, including aspirin, warfarin, barbiturates, diuretics, digoxin, methyl dopa, and several antibiotics. Sulfonamides and iodides have been implicated most frequently. The lesions vary from a few petechiae to massive, generalized petechial eruptions. Mechanisms include development of antibodies to vessel wall components, development of immune complexes, and changes in vessel wall permeability. As soon as the disorder is recognized, the offending drug should be discontinued. No other treatment is necessary.¹⁰

Miscellaneous Causes of Vascular Purpura

Insufficient dietary intake of vitamin C (ascorbic acid) results in *scurvy* and decreased synthesis of collagen, with weakening of capillary walls and the appearance of purpuric lesions.¹⁰ A diagnosis of *purpura simplex* (simple vascular purpura) or *vascular fragility* is made when a cause for purpura cannot be found. The ecchymoses are superficial, bleeding is usually mild, and laboratory test results are most often normal.¹⁰ Cutaneous bleeding and bruising through intact skin has been observed in patients in whom no vascular or platelet dysfunction can be detected. Most such cases involve women with emotional problems, and the bruising is often accompanied by nausea, vomiting, or fever. Evidence for a psychosomatic origin is equivocal. Laboratory test results are invariably normal.¹⁰

SUMMARY

- Inherited qualitative platelet disorders can cause bleeding disorders ranging from mild to severe.
- Bernard-Soulier syndrome is caused by the lack of expression of GP Ib/IX/V complexes on the platelet surface. This receptor complex is responsible for platelet adhesion and its absence results in a severe bleeding disorder.
- Glanzmann thrombasthenia is caused by the lack of expression of GP IIb/IIIa complexes on the platelet surface. This complex is known as the *platelet aggregation receptor*, and its absence is associated with a severe bleeding disorder.
- Storage pool disorders result from the absence of intraplatelet α -granules, dense granules, or both. Platelet dysfunction associated with these disorders is generally mild; bleeding symptoms also are usually mild.
- Aspirin-like effects result from defects in elements of the arachidonic acid metabolic pathway. Platelet dysfunction mimics that seen after aspirin ingestion.
- Deficiencies of several of the receptors for platelet-activating substances have been documented, and bleeding symptoms of varying severity are associated with these deficiencies.
- Drugs are the most common cause of acquired platelet dysfunction, and aspirin is the most frequent culprit. Several new classes of antiplatelet agents with effects different from aspirin are now available and gaining in popularity.
- A variety of pathologic conditions can result in platelet dysfunction and range from hematologic malignancies to kidney disease and liver disease.
- Vascular disorders that result in bleeding are uncommon. There are a few well-recognized inherited disorders, however, such as Ehlers-Danlos syndrome and hereditary hemorrhagic telangiectasia that can result in substantial blood loss.
- Vascular disorders can be acquired, and these are much more common than inherited disorders. Causes range from the effects of aging to drug effects to allergic reaction.

Now that you have completed this chapter, go back and read again the case study at the beginning and respond to the questions presented.

REVIEW QUESTIONS

Answers can be found in the Appendix.

- The clinical presentation of platelet-related bleeding may include all of the following *except*:
 - Bruising
 - Nosebleeds
 - Gastrointestinal bleeding
 - Bleeding into the joints (hemarthroses)
- A defect in GP IIb/IIIa causes:
 - Glanzmann thrombasthenia
 - Bernard-Soulier syndrome
 - Gray platelet syndrome
 - Storage pool disease
- Aspirin ingestion blocks the synthesis of:
 - Thromboxane A_2
 - Ionized calcium
 - Collagen
 - ADP
- Patients with Bernard-Soulier syndrome have which of the following laboratory test findings?
 - Abnormal platelet response to arachidonic acid
 - Abnormal platelet response to ristocetin
 - Abnormal platelet response to collagen
 - Thrombocytosis
- Which of the following is the most common of the hereditary platelet function defects?
 - Glanzmann thrombasthenia
 - Bernard-Soulier syndrome
 - Storage pool defects
 - Multiple myeloma
- A mechanism of antiplatelet drugs targeting GP IIb/IIIa function is:
 - Interference with platelet adhesion to the subendothelium by blocking of the collagen binding site
 - Inhibition of transcription of the GP IIb/IIIa gene
 - Direct binding to GP IIb/IIIa
 - Interference with platelet secretion
- The impaired platelet function in myeloproliferative neoplasms results from:
 - Abnormally shaped platelets
 - Extended platelet life span
 - Increased procoagulant activity
 - Decreased numbers of α - and dense granules
- Which is a *congenital* qualitative platelet disorder?
 - Senile purpura
 - Ehlers-Danlos syndrome
 - Henoch-Schönlein purpura
 - Waldenström macroglobulinemia

9. In uremia, platelet function is impaired by higher than normal levels of:
 - a. Urea
 - b. Uric acid
 - c. Creatinine
 - d. NO
10. The platelet defect associated with increased paraproteins is:
 - a. Impaired membrane activation owing to protein coating
 - b. Hypercoagulability owing to antibody binding and membrane activation
 - c. Impaired aggregation because the hyperviscous plasma prevents platelet-endothelium interaction
 - d. Hypercoagulability because the increased proteins bring platelets closer together, which leads to inappropriate aggregation

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42

Laboratory Evaluation of Hemostasis

George A. Fritsma

OUTLINE

Hemostasis Specimen Collection

Patient Management During Hemostasis Specimen Collection

Hemostasis Specimen Collection Tubes

Hemostasis Specimen Collection Protocol

Specimen Collection Using Syringes and Winged-Needle Sets

Selection of Needles for Hemostasis Specimens

Specimen Collection from Vascular Access Devices

Specimen Collection Using Capillary Puncture

Anticoagulants Used for Hemostasis Specimens

Hemostasis Specimen Management

Hemostasis Specimen Storage Temperature

Hemostasis Specimen Storage Time

Preparation of Hemostasis Specimens for Assay

Platelet Function Tests

Bleeding Time Test for Platelet Function

Platelet Aggregometry and Lumiaggregometry

Testing for Heparin-Induced Thrombocytopenia

Quantitative Measurement of Platelet Markers

Immunoassay for the Anti Platelet Factor 4 (Heparin-Induced Thrombocytopenia) Antibody

Assays for Platelet Activation Markers

OBJECTIVES

After completion of this chapter, the reader will be able to:

1. Properly collect and transport hemostasis blood specimens.
2. Reject hemostasis blood specimens due to clots, short draws, or hemolysis.
3. Prepare hemostasis blood specimens for analysis.
4. Describe the principles of platelet aggregometry.
5. Apply appropriate platelet function tests in a variety of conditions and interpret their results.
6. Diagnose von Willebrand disease and monitor its treatment.
7. Analyze plasma markers of platelet activation platelet factor 4 and β -thromboglobulin.
8. Describe the principle of, appropriately select, and correctly interpret the results of clot-based coagulation screening tests, including activated clotting time, prothrombin time, partial thromboplastin time, and the thrombin clotting time.
9. Interpret clot-based screening test results collectively to reach presumptive diagnoses, and then recommend and perform confirmatory tests.
10. Perform partial thromboplastin time mixing studies to detect factor deficiencies, lupus anticoagulants, and specific factor inhibitors.
11. Describe the principle of, appropriately select, and correctly interpret coagulation factor assays.
12. Describe the principle of and correctly interpret Bethesda titers for coagulation factor inhibitors.
13. Describe the principle of, appropriately select, and correctly interpret tests of fibrinolysis, including assays for D-dimer, plasminogen, plasminogen activators, and plasminogen activator inhibitors.
14. Interpret global coagulation assay tracings: Thromboelastograph and ROTEM.

CASE STUDY

After studying the material in this chapter, the reader should be able to respond to the following case study:

A 54-year-old woman experienced a pulmonary embolism on September 26 and began oral anticoagulant therapy. Monthly PT values were collected to monitor therapy. From October through January, her INR was stable at 2.4, but on February 1, her INR was 1.3. The reduced INR was reported to her physician.

On questioning, the patient reported that there had been no change in her warfarin (Coumadin) dosage or in her diet. She recalled, however, that the phlebotomist had used a tube with a red-and-black stopper. She had thought this to be out of the ordinary and had remarked about it to the phlebotomist, who made no response. The medical laboratory practitioner who had performed the PT assay reexamined the blood specimen and saw that it was in a blue-topped tube.

1. What did the phlebotomist do?
2. What was the consequence of this action?
3. What else could cause an unexpectedly short PT?

OUTLINE—cont'd**Clot-Based Plasma Procoagulant Screens**

Prothrombin Time
 Partial Thromboplastin Time
 Partial Thromboplastin Time
 Mixing Studies
 Thrombin Clotting Time
 Reptilase Time
 Russell Viper Venom

Coagulation Factor Assays

Fibrinogen Assay
 Single-Factor Assays Using
 the Partial Thromboplastin
 Time Test
 Bethesda Titer for Anti Fac-
 tor VIII Inhibitor
 Single-Factor Assays Using
 the Prothrombin Time Test
 Factor XIII Assay

Tests of Fibrinolysis

Quantitative D-Dimer Immu-
 noassay
 Fibrin Degradation Product
 Immunoassay
 Plasminogen Chromogenic
 Substrate Assay
 Tissue Plasminogen Activa-
 tor Assay
 Plasminogen Activator In-
 hibitor-1 Assay

Global Coagulation Assays**HEMOSTASIS SPECIMEN COLLECTION**

Most hemostasis laboratory procedures are performed on venous whole blood collected by venipuncture and mixed 9:1 with a 3.2% solution of sodium citrate anticoagulant. The specimen is maintained as well-mixed whole blood for platelet function testing or centrifuged to provide platelet-poor plasma (PPP) for other procedures. Phlebotomists, patient care technicians, nurses, medical laboratory practitioners, and other health personnel who collect blood specimens must adhere closely to published protocols for specimen collection and management. The nursing or laboratory supervisor is responsible for the current validity of specimen collection and handling protocols and ensures that personnel employ approved techniques.¹

Patient Management During Hemostasis Specimen Collection

Patients need not fast, but they should avoid vigorous activities and should rest quietly for 30 minutes prior to collection for hemostasis testing. Little additional preparation is necessary; however, there are numerous drugs that affect the outcomes of coagulation tests. For example, aspirin suppresses most platelet function, and Coumadin (warfarin) reduces the activities of factor II (prothrombin), factor VII, factor IX, factor X, protein C, protein S, and protein Z and prolongs the prothrombin time (PT) test. The phlebotomist should attempt to record all drugs the patient is currently taking, and patients should be instructed by their physicians to discontinue drugs that may interfere with coagulation test results before testing.

Phlebotomists may manage patients using standard protocols for identification, cleansing, tourniquet use, and venipuncture (Chapter 3). If there is a reason to anticipate excessive bleeding—for instance, if the patient has multiple bruises or mentions a tendency to bleed—the phlebotomist should extend the time for observing the venipuncture site from 1 to 5 minutes and should apply a pressure bandage before dismissing the patient.

Hemostasis Specimen Collection Tubes

Most hemostasis specimens are collected in *plastic blue-stopper* (blue-top, blue-closure) *sterile evacuated blood collection tubes* containing a measured volume of 0.105 to 0.109 M (3.2%) buffered sodium citrate anticoagulant.² Tubes of uncoated soda-lime glass are unsuitable because their negative surface charge activates platelets and plasma procoagulants. *Siliconized* (plastic-coated) glass tubes are available, but their use is waning because of concern for potential breakage, with consequent risk of exposure to bloodborne pathogens.³

Hemostasis Specimen Collection Protocol

Laboratory directors typically prefer evacuated blood collection tube systems for hemostasis blood collections; however, many directors may require syringe collection and initial “discard tubes” in special circumstances. [Table 42-1](#) provides a list of collection errors.

- If the hemostasis specimen is part of a *series of tubes* to be filled from a single venipuncture site, it must be collected *first* or immediately after a *nonadditive tube*. The hemostasis tube may not immediately follow a tube that contains heparin (green stopper), ethylenediamine tetraacetic acid (EDTA,

TABLE 42-1 Hemostasis Specimen Collection Errors That Require Collection of a New Specimen

Error	Comments
Short draw	Whole-blood volume less than 90% of required volume or less than manufacturer specified minimum.
Clot in specimen	Each specimen must be visually inspected prior to centrifugation; the presence of even a small clot requires that the specimen be recollected.
Visible hemolysis	Hemolysis, pink or red plasma indicates in vitro activation of platelets and coagulation. Results are unreliable.
Lipemia or icterus	Optical instruments may not measure clots in cloudy or highly colored specimens, especially chromogenic substrate methods. The practitioner must employ a mechanical instrument.
Prolonged tourniquet application	Stasis elevates the concentration of von Willebrand factor and factor VIII; falsely decreases fibrinolytic parameters; and falsely shortens clot-based test results.
Specimen storage at 1° C to 6° C	Storage at refrigerator temperatures causes precipitation of large von Willebrand factor multimers, activation of coagulation factor VII, and destroys platelet integrity.
Specimen storage at more than 25° C	Storage at above standard room temperature causes coagulation factors V and VIII to deteriorate.

lavender stopper), sodium fluoride (gray stopper), or clot-promoting *silica* particles as contained in plastic red-topped or serum separator (gel) tubes. These additives may become transferred to the hemostasis specimen on the stopper needle and invalidate all hemostasis test results. Nonadditive tubes include red-topped glass tubes and clear-topped or red-and-gray marble-topped tubes. If nonadditive tubes are unavailable, the phlebotomist may use and discard a preliminary blue-topped tube.⁴ Some hemostasis laboratory directors specify that a nonadditive tube be collected and discarded prior to the hemostasis specimen when the specimen is intended for platelet function studies or specialized coagulation assays. Their purpose is to ensure the absence of tissue contaminants in the specimen.

- The ratio of whole blood to anticoagulant must be 9 parts blood to 1 part anticoagulant. Evacuated tubes are designed so that the negative internal pressure draws the correct volume of blood from the vein. Collection tube manufacturers indicate the allowable range of collection volume error in package inserts and provide a minimum volume line on each tube. In most cases, the volume of blood collected must be within 90% of the calibrated volume. A *short draw*—that is, a specimen with a smaller volume than the minimum specified by the manufacturer—generates erroneously prolonged clot-based coagulation test results because the excess anticoagulant relative to blood volume neutralizes test reagent calcium.⁵ Short-draw specimens are consistently discarded, and a fresh specimen is collected from the patient. Most plastic blue-topped tubes collect 2.7 mL of whole blood; the smaller the collection tube, the narrower the tolerance for short draws.
- When specimens are collected using *winged-needle butterfly sets*, the phlebotomist must compensate for the internal volume of the tubing, which is usually 12 inches long and contains approximately 0.5 mL of air. The phlebotomist must first collect and discard a nonadditive tube or an identical blue-topped tube. This step ensures that the needle set tubing is filled with fresh patient blood before the hemostasis specimen is collected.⁶
- *Clotted specimens* are useless for hemostasis testing, even if the clot is small. A few seconds after collection, the phlebotomist must gently invert the specimen at least *five times* to mix the blood with the anticoagulant and prevent clot formation. If possible, the medical laboratory practitioner must visually examine for clots just before centrifugation and testing. Many coagulometers are equipped to detect the presence of clots. Clotted specimens are discarded, and a new specimen is collected from the patient.
- Excessive specimen agitation causes hemolysis (RBC rupture), procoagulant activation, and platelet activation. The phlebotomist must *never shake the tube*. The test results from visibly hemolyzed specimens are unreliable, and the specimen must be recollected.⁷
- Excess needle manipulation may promote the release of procoagulant substances from the skin and connective tissue, which contaminate the specimen and cause clotting

factor activation. Consequently, test results from specimens collected during a *traumatic* venipuncture may be falsely shortened and unreliable.⁸

- During blood collection, the phlebotomist must remove the tourniquet within 1 minute of its application to avoid blood *stasis*.⁹ Stasis is a condition in which venous flow is slowed. Stasis results in the local accumulation of coagulation factor VIII and von Willebrand factor (VWF), which may result in false shortening of clot-based coagulation test results.

Specimen Collection Using Syringes and Winged-Needle Sets

Managers of many hemostasis specialty laboratories insist that specimens from patients with difficult venous access and patients whose veins are small, fragile, or scarred by repeated venipunctures be collected by syringe. Additionally, in an effort to reduce the activation of platelets and coagulation, specimens for specialized tests such as platelet aggregometry are collected by syringe. Many hemostasis laboratories employ medical laboratory practitioners and phlebotomists who are specially trained in specimen collection to ensure the integrity of the specimen. The use of syringes presents additional needle-stick risk to the phlebotomist, so careful training and handling are essential.¹⁰

The phlebotomist selects sterile syringes of 20 mL capacity or less with nonthreaded Luer-slip hubs. The phlebotomist assembles syringes, a winged needle set (Figure 42-1), a tubing clamp, and standard venipuncture materials. The phlebotomist then uses the following protocol:

1. Use standard patient identification and standard blood specimen management precautions (Chapter 3).
2. Most syringes are delivered with the plunger withdrawn about 1 mm from the end of the barrel. Move the plunger outward and inward within the barrel. Expel all air from the barrel and affix the needle set to the Luer-slip hub.
3. Cleanse the venipuncture site, affix the tourniquet, and insert the winged needle. Immobilize the needle set by loosely taping the tube to the arm about 2 inches from the needle.
4. Fill the syringe using a gentle, even pressure.
5. Place the syringe on a clean surface and clamp the tubing with a hemostat near the needle hub.
6. Remove the first syringe and discard to avoid tissue contamination of the hemostasis specimen. The phlebotomist may use this specimen for chemistry or other tests. Attach a second syringe for collection of the hemostasis specimen; release the clamp and fill the second syringe. Repeat if needed.
7. Replace the clamp, remove the needle set, and immediately activate the needle cover.

After seeing to the patient's welfare, the phlebotomist cautiously transfers the blood specimen to sealed evacuated tubes by affixing a safety transfer device. The specimen is allowed to flow gently down the side of the tube. The specimen is not pushed forcibly into the tube, because agitation causes hemolysis and platelet activation. The phlebotomist must transfer the specimen within a few seconds of the time the syringe is filled, and the tube

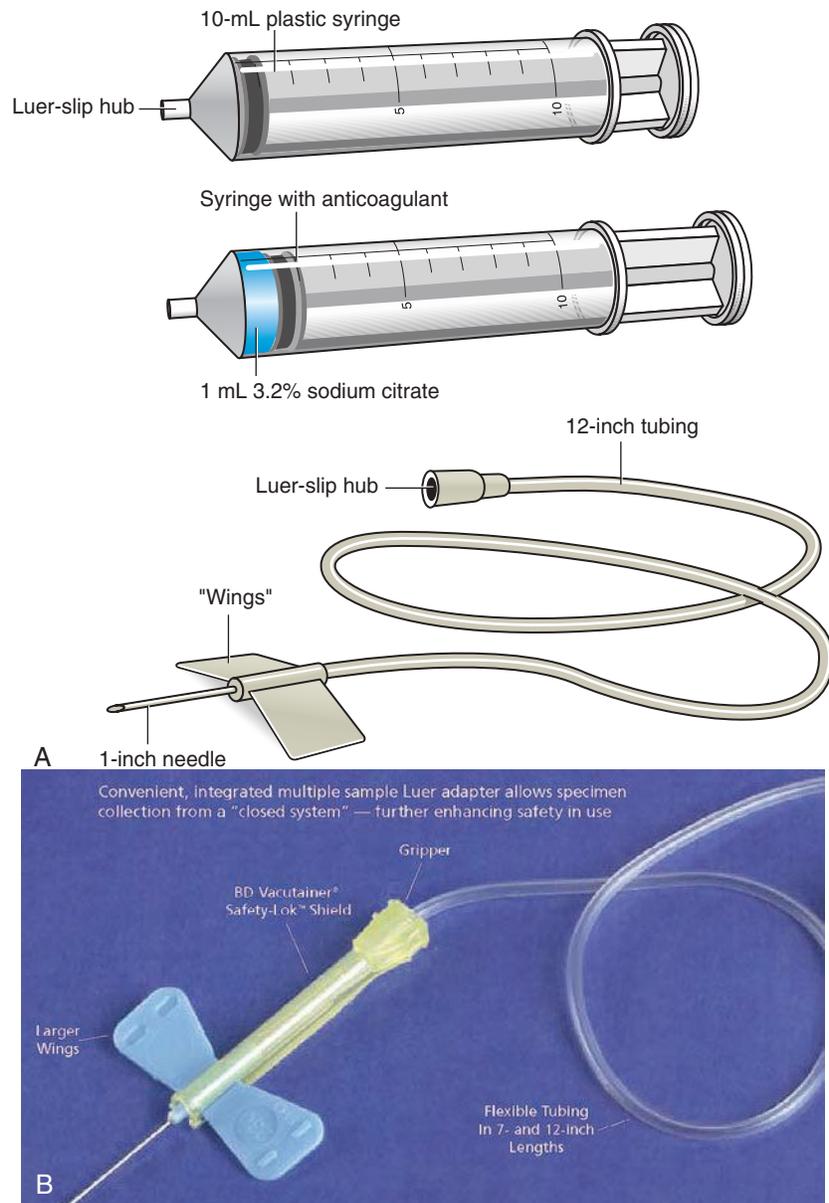


Figure 42-1 **A**, Winged needle set and syringe for collecting special hemostasis specimens. The phlebotomist may use the option of drawing the desired volume of anticoagulant into the syringe prior to blood collection. **B**, Winged needle illustrating needle-covering safety interlock.

must be gently inverted at least five times. The specimen volume must be correct for the proper ratio of blood to sodium citrate.

Selection of Needles for Hemostasis Specimens

Whether evacuated collection tubes or syringes are used, the bore of the needle should be sufficient to prevent hemolysis and activation of platelets and plasma procoagulants. If the overall specimen is 25 mL or less, a 20- or 21-gauge thin-walled needle is used (Table 42-2). For a larger specimen, a 19-gauge needle is required. A 23-gauge needle is acceptable for pediatric patients or patients whose veins are small, but the negative collection pressure must be reduced. All needles provide safety

closures that either cover or blunt the needle immediately after completion of the venipuncture.

Specimen Collection from Vascular Access Devices

Blood specimens may be drawn from heparin or saline locks, ports in intravenous lines, peripherally inserted central catheters (PICC tubes), central venous catheters, or dialysis catheters. Vascular access device management requires strict adherence to protocol to ensure sterility, prevent emboli, and prevent damage to the device. Personnel must be trained and must recognize the signs of complications and take appropriate action. Institutional protocol may limit vascular

TABLE 42-2 Selection of Needles for Hemostasis Specimens

Application	Preferred Needle Gauge and Length
Adult with good veins, specimen ≤ 25 mL	20 or 21 gauge, thin-walled, 1.0 or 1.25 inches long
Adult with good veins, specimen ≥ 25 mL	19 gauge, 1.0 or 1.25 inches long
Child or adult with small, friable, or hardened veins	23 gauge, winged-needle set; apply minimal negative pressure
Transfer of blood from syringe to tube	19 gauge, slowly inject through tube closure
Syringe with winged-needle set	20, 21, or 23 gauge, thin-walled; use only for small, friable, or hardened veins or specialized coagulation testing

access device blood collection to physicians and nurses. Before blood is collected for hemostasis testing, the line must be flushed with 5 mL of saline, and the first 5 mL of blood, or six times the volume of the tube, must be collected and discarded. The phlebotomist *must not flush with heparin*. Blood is collected into a syringe and transferred to an evacuated tube as described in the prior section on hemostasis specimen collection with syringes and winged needle sets.¹¹

Specimen Collection Using Capillary Puncture

Several near-patient testing (point-of-care) coagulometers (Chapter 44) generate PT results from a specimen consisting of 10 to 50 μ L of whole blood. These instruments are designed to test either anticoagulated venous whole blood or capillary (finger-stick) blood and represent a significant convenience to patients and to anticoagulation clinics.¹² Many are designed for patient self-testing and pediatric or neonatal testing, and laboratory practitioners are often charged with training patients in proper capillary puncture technique.¹³

Capillary specimen punctures are made using sterile spring-loaded lancets designed to make a cut of standard depth and width, while avoiding injury (Chapter 3). The phlebotomist or patient selects and cleanses the middle or fourth (ring) finger and activates the device so that it produces a puncture that is just off-center of the fingertip and perpendicular to the fingerprint lines. After wiping away the first drop of blood, which is likely to be contaminated by tissue fluid, the phlebotomist places the collection device directly adjacent to the free-flowing blood and allows the device to fill. The phlebotomist wipes excess blood from the outside of the device and introduces it to the coagulometer to complete the assay. The phlebotomist then presses a gauze pad to the wound and instructs the patient to maintain pressure until bleeding ceases. The phlebotomist then provides a spot bandage to cover the wound.

The key to accurate PT measurement is a free-flowing puncture. Often it is necessary for the phlebotomist to warm the patient's hand to increase blood flow to the fingertips. Blood collection device distributors provide dry, disposable warming devices for this purpose. The phlebotomist avoids squeezing ("milking") the finger, because this renders the blood specimen inaccurate by raising the concentration of tissue fluid relative to blood cells.¹⁴

Anticoagulants Used for Hemostasis Specimens

Sodium Citrate (Primary Hemostasis Anticoagulant)

The anticoagulant used for hemostasis testing is buffered 3.2% (0.105 to 0.109 M) sodium citrate, $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 2\text{H}_2\text{O}$, molecular weight 294.1 Daltons. Sodium citrate binds calcium ions to prevent coagulation, and the buffer stabilizes specimen pH as long as the tube stopper remains in place.¹⁵

The anticoagulant solution is mixed with blood to produce a 9:1 ratio: 9 parts whole blood to 1 part anticoagulant. In most cases, 0.3 mL of anticoagulant is mixed with 2.7 mL of whole blood, which are the volumes in the most commonly used evacuated plastic collection tubes, but any volumes are valid, provided that the 9:1 ratio is maintained. The ratio yields a final citrate concentration of 10.5 to 10.9 mM of anticoagulant in whole blood.¹⁶ Some laboratory practitioners prepare specimen tubes locally for special hemostasis testing.

Adjustment of Sodium Citrate Volume for Elevated Hematocrits

The 9:1 blood-to-anticoagulant ratio is effective, provided the patient's hematocrit is 55% or less. In polycythemia, the decrease in plasma volume relative to whole blood unacceptably raises the anticoagulant-to-plasma ratio, which causes falsely prolonged results for clot-based coagulation tests. The phlebotomist must provide tubes with relatively reduced anticoagulant volumes for collection of blood from a patient whose hematocrit is known to be 55% or higher. The amount of anticoagulant needed may be computed for a 5 mL total specimen volume by using the graph in Figure 42-2 or the following formula, which is valid for any total volume:

$$C = (1.85 \times 10^{-3})(100 - H)V$$

where C is the volume of sodium citrate in milliliters, V is volume of whole blood-sodium citrate solution in milliliters, and H is the hematocrit in percent.

For example, to collect 3 mL of blood and anticoagulant mixture from a patient who has a hematocrit of 65%, calculate the volume of sodium citrate as follows:

$$\begin{aligned} C &= (1.85 \times 10^{-3})(100 - 65\%) \times 3.0 \text{ mL} \\ C &= (1.85 \times 10^{-3})(35\%) \times 3.0 \text{ mL} \\ C &= 0.19 \text{ mL of 3.2\% sodium citrate} \end{aligned}$$

Remove the stopper from the blue closure collection tube, pipette and discard 0.11 mL from the 0.3 mL of anticoagulant, leaving 0.19 mL. Collect blood in a syringe and transfer

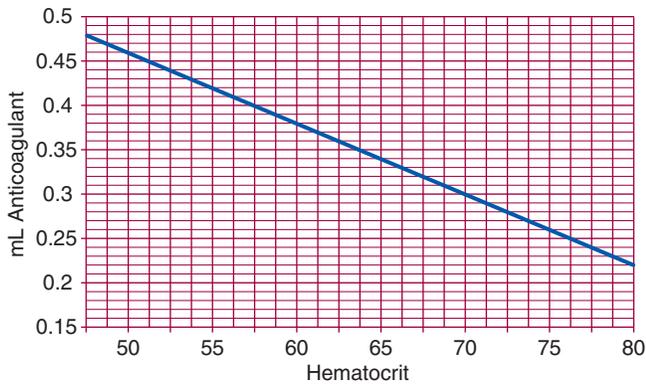


Figure 42-2 Graph for computing the volume of anticoagulant in a 5.0 mL specimen when the patient's hematocrit is 55% or greater. (From Ingram GIC, Brozovic M, Slater NGP: *Bleeding disorders, investigations, and management*, ed 2, Oxford, 1982, Blackwell, pp. 244-245.)

2.81 (2.8) mL of blood to the tube, replace the stopper, and immediately mix by gently inverting four times. Alternatively, the laboratory practitioner can prepare for collection of 10 mL of blood and anticoagulant solution in a 12 mL centrifuge tube as follows:

$$C = (1.85 \times 10^{-3})(35\%) \times 10.0 \text{ mL}$$

$$C = 0.64 \text{ mL of 3.2\% sodium citrate}$$

In this instance, 0.64 mL of sodium citrate is pipetted into the tube, and 9.36 (9.4) mL of whole blood is transferred from the collection syringe. There is no evidence suggesting a need for increasing the volume of anticoagulant for specimens from patients with anemia, even when the hematocrit is less than 20%.

Other Anticoagulants Used for Hemostasis Specimens

EDTA-anticoagulated specimens are not used for coagulation testing because calcium ion chelation by EDTA is irreversible, interfering with coagulation assays.¹⁷ Calcium ion chelation with citrate, on the other hand, is reversed with the addition of calcium. EDTA is the anticoagulant used in collecting specimens for complete blood counts, including platelet counts.

EDTA may be required for specimens used for *molecular* diagnostic testing, such as testing for *factor V Leiden mutation* or the *prothrombin G20210A mutation*. Likewise, acid citrate dextrose (ACD, yellow stopper) and dipotassium EDTA (K₂EDTA) with gel (white stopper) tubes may be used for molecular diagnosis, as specified by institutional protocol. Heparinized specimens have never been validated for use in plasma coagulation testing but may be necessary in cases of platelet satellitosis (satellitism) as a substitute for specimens collected in EDTA or sodium citrate. *Citrate theophylline adenosine dipyridamole* (CTAD, blue stopper) tubes are used to halt in vitro platelet or coagulation activation for specialty assays such as those for the platelet activation markers *platelet factor 4* (PF4) and platelet surface membrane *P-selectin* (measured by flow cytometry) or the coagulation activation markers *prothrombin fragment 1+2* and *thrombin-antithrombin complex*.

HEMOSTASIS SPECIMEN MANAGEMENT

Hemostasis Specimen Storage Temperature

Sodium citrate-anticoagulated whole blood specimens are placed in a rack and allowed to stand in a vertical position with the stopper intact and uppermost. The pH remains constant as long as the specimen is sealed. Specimens are maintained at 18° C to 24° C (ambient temperature), never at refrigerator temperatures (Table 42-3). Storage at 1° C to 6° C activates factor VII, destroys platelet activity through uncontrolled activation, and causes the cryoprecipitation of large VWF multimers.^{18,19} Also, specimens should never be stored at temperatures greater than 24° C because heat causes deterioration of coagulation factors V and VIII.

Hemostasis Specimen Storage Time

Specimens collected for PT testing may be held at 18° C to 24° C and tested within 24 hours of the time of collection. Specimens collected for partial thromboplastin time (PTT) testing also may be held at 18° C to 24° C, but they must be tested within 4 hours of the time of collection, provided that the specimen does not contain unfractionated heparin anticoagulant. If a patient is getting unfractionated heparin therapy, specimens for PTT testing must be centrifuged within 1 hour of the time of collection, and the plasma, which should be PPP, must be tested within 4 hours of the time of collection.²⁰

TABLE 42-3 Hemostasis Specimen Storage Times and Temperatures

Application	Temperature	Time
PT with no unfractionated heparin present in specimen	18–24° C	24 hours
PTT with no unfractionated heparin present in specimen	18–24° C	4 hours
PTT for monitoring unfractionated heparin therapy	18–24° C	Separate within 1 hour, test within 4 hours
PT when unfractionated heparin is present in specimen	18–24° C	Separate within 1 hour, test within 4 hours
Factor assays	18–24° C	4 hours
Optical platelet aggregometry using platelet-rich plasma	18–24° C	Wait 30 min after centrifugation, test within 4 hours of collection
Whole-blood aggregometry	18–24° C	Test within 3 hours of collection
Storage in household freezer	–20° C	2 weeks
Storage for 6 months	–70° C	6 months

PT, Prothrombin time; PTT, partial thromboplastin time.

Preparation of Hemostasis Specimens for Assay

Whole-Blood Specimens Used for Platelet Aggregometry

Blood for whole-blood platelet aggregometry or lumiaggregometry must be collected with 3.2% sodium citrate and held at 18° C to 24° C until testing. Chilling destroys platelet activity. Aggregometry should be started immediately and must be completed within 4 hours of specimen collection. The practitioner mixes the specimen by gentle inversion, checks for clots just before testing, and rejects specimens with clots. Most specimens for whole-blood aggregometry are mixed 1:1 with normal saline before testing, although if the platelet count is less than 100,000/ μ L the specimen is tested undiluted.²¹

Platelet-Rich Plasma Specimens Used for Platelet Aggregometry

Light-transmittance (optical) platelet aggregometers are designed to test platelet-rich plasma (PRP), plasma with a platelet count of 200,000 to 300,000/ μ L. Sodium citrate-anticoagulated blood is first checked visually for clots and then centrifuged at 50 *xg* for 30 minutes with the stopper in place to maintain the pH. The supernatant PRP is transferred by a plastic pipette to a clean plastic tube, and the tube is sealed and stored at 18° C to 24° C (ambient temperature) until the test is begun. PRP-based light-transmittance aggregometry is initiated no less than 30 minutes after the specimen is centrifuged and completed within 4 hours of the time of collection. To produce sufficient PRP, the original specimen must measure 9 to 12 mL of whole blood. Light-transmittance aggregometry is unreliable when the patient's whole-blood platelet count is less than 100,000/ μ L.

Platelet-Poor Plasma Required for Clot-Based Testing

Clot-based plasma coagulation tests require PPP-plasma with a platelet count of less than 10,000/ μ L.²² Sodium citrate-anticoagulated whole blood is centrifuged at 1500 *xg* for 15 minutes in a *swinging bucket* centrifuge to produce supernatant PPP. Alternatively, the *angle-head* StatSpin Express 2 (Iris Sample Processing, Inc., Westwood, MA) generates 4400 *xg* and can produce PPP within 3 minutes. Both make it possible for automated coagulometers to sample from the supernatant plasma of the primary blood collection tube. The advantage of the slower swinging bucket centrifuge head is that it produces a straight, level plasma-blood cell interface, whereas angle-head centrifuge heads cause platelets to adhere to the side of the tube. If the "angle-spun" tube is allowed to stand, the adherent platelets drift back into the plasma and release granule contents. Each hemostasis laboratory manager establishes the correct centrifugation speed and times for the local laboratory. Centrifugation must yield PPP from specimens with high initial platelet counts.

In the special hemostasis laboratory the manager may choose a *double-spin* approach. The primary tube is centrifuged using a swinging bucket centrifuge, and the plasma is transferred to a secondary plastic tube, which is labeled and centrifuged again. The double-spin approach may be used to produce PPP with a plasma platelet count of less than 5000/ μ L, which some labora-

tory directors prefer for lupus anticoagulant (LA) testing and for preparation of frozen plasma.

The presence of greater than 10,000 platelets/ μ L in plasma affects clot-based test results. Platelets are likely to become activated in vitro and release the membrane phospholipid *phosphatidylserine*, which triggers plasma coagulation and neutralizes LA if present, interfering with LA testing. Platelets also secrete fibrinogen, factors V and VIII, and VWF (Chapter 13). These may desensitize PT and PTT assays and interfere with clot-based coagulation assays. In addition, platelets release platelet factor 4 (PF4), a protein that binds and neutralizes therapeutic heparin in vitro, falsely shortening the PTT and interfering with heparin management.

The hemostasis laboratory manager arranges to perform plasma platelet counts on coagulation plasmas at regular intervals to ensure that they are consistently platelet poor. Many managers select 10 to 12 specimens from each centrifuge every 6 months, perform plasma platelet counts, and document that their samples remain appropriately platelet poor, even if the initial platelet count is elevated.

Laboratory practitioners inspect hemostasis plasmas for hemolysis (red), lipemia (cloudy, milky), and icterus (golden yellow from bilirubin). Visible hemolysis implies platelet or coagulation pathway activation. Visibly hemolyzed specimens are rejected, and new specimens must be obtained. Lipemia and icterus may affect the end-point results of optical coagulation instruments. The hemostasis laboratory manager may choose to maintain a separate mechanical end-point coagulometer to substitute for the optical instrument if the specimen is too cloudy for optical determinations. Conversely, some optical instruments detect and compensate for lipemia and icterus via spectrophotometric analysis.²³

Specimen Storage

Specimens for PT assay only may be held uncentrifuged at 18° C to 24° C for up to 24 hours, provided the tubes remain closed. Likewise, specimens for PTT measurement may be held uncentrifuged for up to 4 hours. However, specimens from patients receiving unfractionated heparin collected for PTT heparin monitoring must be centrifuged, and the supernatant PPP must be sampled or transferred within 1 hour to avoid false shortening of the PTT as platelet granule PF4 neutralizes the heparin.

If the hemostasis test cannot be completed within the prescribed interval, the laboratory practitioner must immediately centrifuge the specimen. The supernatant PPP must be transferred by plastic pipette to a plastic freezer tube (non-siliconized glass materials are never used with plasma handling as it activates the coagulation cascade), sealed, and frozen and may be stored at -20° C for up to 2 weeks or at -70° C for up to 6 months. At the time the test is performed, the specimen must be thawed rapidly at 37° C, mixed well, and tested within 1 hour of the time it is removed from the freezer. If it cannot be tested immediately, the specimen may be stored at 1° C to 6° C for 2 hours after thawing. To avoid cryoprecipitation of VWF, specimens may not be frozen and thawed more than once.

PLATELET FUNCTION TESTS

Platelet function tests are designed to detect qualitative (functional) platelet abnormalities in patients who are experiencing the symptoms of mucocutaneous bleeding (Chapter 41). A platelet count is performed, and the blood film is reviewed before platelet function tests are begun, because thrombocytopenia is a common cause of hemorrhage (Chapter 40).²⁴ Qualitative platelet abnormalities are suspected only when bleeding symptoms are present and the platelet count exceeds 50,000/ μ L.

Although hereditary platelet function disorders are rare, acquired defects are common.²⁵ Acquired platelet defects are associated with liver disease, renal disease, myeloproliferative neoplasms, myelodysplastic syndromes, myeloma, uremia, autoimmune disorders, anemias, and drug therapy. Platelet morphology is often a clue; for instance, in Bernard-Soulier syndrome, the blood film reveals mild thrombocytopenia and large gray platelets (Figure 41-3). Similarly, the presence of large platelets on the blood film associated with elevated mean platelet volume often indicates rapid platelet turnover, such as what occurs in *immune thrombocytopenic purpura* or *thrombotic thrombocytopenic purpura*. Giant or dysplastic platelets are seen in myeloproliferative neoplasms, acute leukemia, and myelodysplastic syndromes.

Bleeding Time Test for Platelet Function

The *bleeding time* test was the original test of platelet function, although it is now largely replaced by near-patient analysis of platelet function using the PFA-100 (Siemens Healthcare Diagnostics, Inc., Deerfield, IL), the Multiplate (DiaPharma, West Chester, OH), or platelet aggregometry.²⁶ To perform the test, the phlebotomist uses a lancet to make a small, controlled puncture wound and records the duration of bleeding, comparing the results with the universally accepted reference interval of 2 to 9 minutes. The bleeding time test was first described by Duke²⁷ in 1912 and modified by Ivy²⁸ in 1941. In 1978 some standardization was attempted. A blood pressure cuff was inflated to 40 mm Hg, a calibrated spring-loaded lancet (Surgicutt Bleeding Time Device; International Technidyne Corp., Edison, NJ) was triggered on the volar surface of the forearm a few inches distal to the antecubital crease, and the resulting wound was blotted every 30 seconds with filter paper until bleeding stopped.^{29,30}

A prolonged bleeding time could theoretically signal a functional platelet disorder such as von Willebrand disease (VWD) or a vascular disorder such as scurvy or vasculitis, and was a characteristic result of therapy with aspirin and other nonsteroidal anti-inflammatory drugs (NSAIDs). Measurement of the bleeding time was often requested by surgeons at admission in an attempt to predict surgical bleeding, but a series of studies in the 1990s revealed that the test has inadequate predictive value. The bleeding time is affected by the nonplatelet variables of intracapillary pressure, skin thickness at the puncture site, and size and depth of the wound, all of which interfere with accurate interpretation of the test results. Owing to its poor predictive value for bleeding and its tendency to scar the forearm, use of the bleeding time assay has been discontinued at most institutions.

Platelet Aggregometry and Lumiaggregometry

Functional platelets *adhere* to subendothelial collagen, *aggregate* with one another, and *secrete* the contents of their α -granules and dense granules (Chapter 13). Normal adhesion requires intact platelet membranes and functional plasma VWF. Normal aggregation requires that platelet membranes and platelet activation pathways are intact, that the plasma fibrinogen concentration is normal, and that normal secretions are released from platelet granules. Platelet adhesion, aggregation, and secretion are assessed using *in vitro* platelet aggregometry.

An aggregometer is an instrument designed to measure platelet aggregation in a suspension of citrated whole blood or PRP. Specimens are collected and managed in compliance with standard laboratory protocol as described in the section entitled Preparation of Hemostasis Specimens for Assay, and maintained at ambient temperature (18° C to 24° C) until testing begins. Specimens for PRP-based light-transmittance aggregometry must stand undisturbed for 30 minutes after centrifugation while the platelets regain their responsiveness. Specimens for impedance whole blood aggregometry are diluted 1:1 with normal saline and tested immediately. Specimens must be tested within 4 hours of collection to avoid spontaneous *in vitro* platelet activation and loss of normal activity. Platelet aggregometry is a high-complexity laboratory test requiring a skilled, experienced operator.

Platelet Aggregometry Using Platelet-Rich Plasma

PRP aggregometry is performed using a specialized photometer called a *light-transmittance aggregometer* (PAP-8E Platelet Aggregation Profiler; Bio/Data Corp., Horsham, PA).³¹ After calibrating the instrument in accordance with manufacturer instructions, the operator pipettes the PRP to instrument-compatible cuvettes, usually 500 μ L; drops in one clean plasticized stir bar per sample; places the cuvettes in incubation wells; and allows the samples to warm to 37° C for 5 minutes. The operator then transfers the first cuvette, containing specimen and stir bar, to the instrument's reaction well and starts the stirring device and the recording computer. The stirring device turns the stir bar at 800 to 1200 rpm, a gentle speed that keeps the platelets in suspension. The instrument directs focused light through the sample cuvette to a photodetector (Figure 42-3). As the PRP is stirred, the recorder tracing first stabilizes to generate a baseline, near 0% light transmittance. After a few seconds, the operator pipettes an agonist (aggregating agent) directly into the sample to trigger aggregation. In a normal specimen, after the agonist is added, the shape of the suspended platelets changes from discoid to spherical, and the intensity of light transmittance initially (and briefly) decreases, then increases in proportion to the degree of shape change. Percent light transmittance is monitored continuously and recorded (Figure 42-4). As platelet aggregates form, more light passes through the PRP, and the tracing begins to move toward 100% light transmittance. Platelet function deficiencies are reflected in diminished or absent aggregation; many laboratory directors choose 40% aggregation as the lower limit of normal.

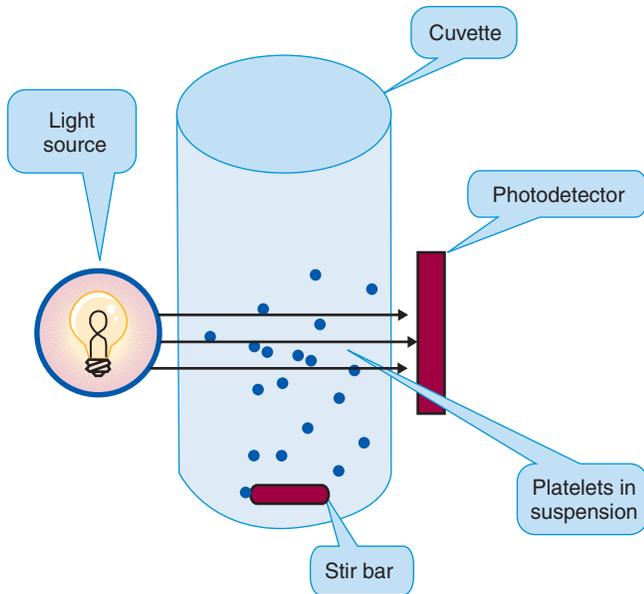


Figure 42-3 Analysis of platelet-rich plasma in an optical aggregometer. Desired platelet count is approximately 200,000/ μL . Platelets are maintained in suspension by a magnetic stir bar turning at 800 to 1200 rpm. (Courtesy Kathy Jacobs, Chrono-log Corp., Havertown, PA.)

Whole-Blood Platelet Aggregometry

In whole-blood platelet aggregometry, platelet aggregation is measured by electrical impedance using a 1:1 saline-whole blood suspension (Model 700 Whole Blood/Optical Lumi-Aggregometer; Chrono-log Corp., Havertown, PA).³² The operator pipettes aliquots of properly mixed whole blood to cuvettes and adds equal volumes of physiologic saline. Suspension volume may be 300 to 500 μL . The operator drops in one stir bar per cuvette and places the cuvettes in 37° C incubation wells for 5 minutes. The operator transfers the first cuvette to a reaction well, pipettes an agonist directly into the specimen, and suspends a pair of low-voltage cartridge-mounted disposable direct current (DC) electrodes in the mixture. As aggregation occurs, platelets adhere to the electrodes and one another, impeding the DC current (Figure 42-5). The rise in impedance, which is directly proportional to platelet aggregation, is amplified and recorded by instrument circuitry. A whole-blood aggregometry tracing closely resembles a PRP-based light-transmittance aggregometry tracing, as shown in Figure 42-4.

Platelet Lumiaggregometry

The Chrono-log Whole Blood/Optical Lumi-Aggregometer may also be used for simultaneous measurement of platelet aggregation

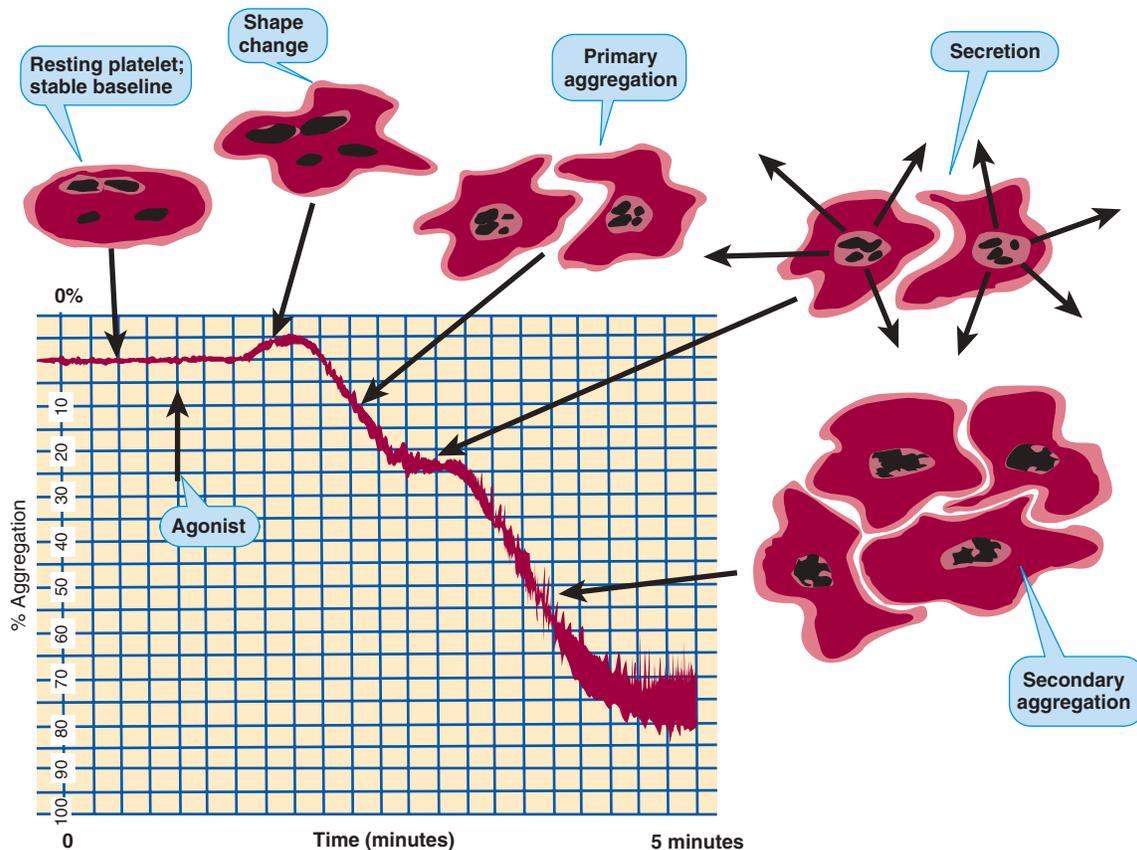


Figure 42-4 Optical aggregometry tracing showing five phases of platelet aggregation: baseline at 0% aggregation, shape change after the addition of the agonist, primary aggregation, release of adenosine diphosphate and adenosine triphosphate, and second-wave aggregation that forms large clumps. The % aggregation is measured by amount of light transmittance through the test sample. (Courtesy Kathy Jacobs, Chrono-log Corp., Havertown, PA.)

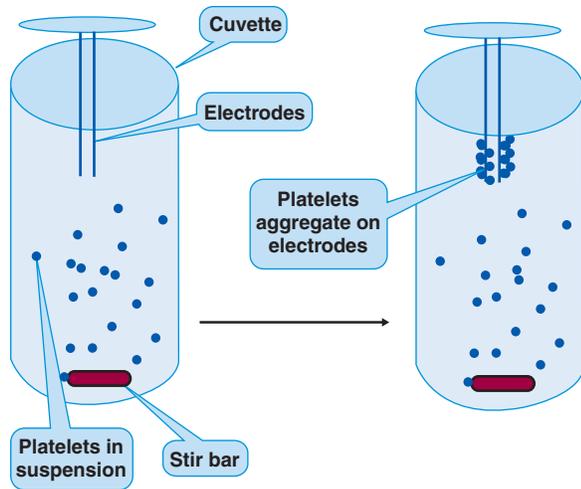


Figure 42-5 In whole-blood platelet aggregometry, aggregating platelets form a layer on the electrodes and the platelet layer impedes current. Resistance (in ohms) is proportional to aggregation, and a tracing is provided that resembles the tracing obtained using optical aggregometry. (Courtesy Kathy Jacobs, Chrono-log Corp., Havertown, PA.)

and the secretion of adenosine triphosphate (ATP) from activated platelet dense granules.³³ The procedure for *lumiaggregometry* differs little from that for conventional aggregometry and simplifies the diagnosis of platelet dysfunction.³⁴ As ATP is released, it oxidizes a firefly-derived luciferin-luciferase reagent (Chrono-lume; Chrono-log Corp.) to generate cold chemiluminescence proportional to the ATP concentration. A photodetector amplifies the luminescence, which is recorded as a second tracing on the aggregation report.³⁵

Lumiaggregometry may be performed using whole blood or PRP.³⁶ To perform lumiaggregometry, the operator adds an ATP standard to the first sample, then adds luciferin-luciferase and tests for full luminescence. The operator then adds luciferin-luciferase and an agonist to the second sample; the instrument monitors for aggregation and secretion simultaneously. *Thrombin* is typically the first agonist used because thrombin induces full secretion. The luminescence induced by thrombin is measured, recorded, and used for comparison with the luminescence produced by the additional agonists. Normal secretion induced by agonists other than thrombin produces luminescence at a level of about 50% of that resulting from thrombin (Table 42-4). Figure 42-6 depicts simultaneous aggregation and secretion responses to thrombin; Figure 42-7 is a scanning electron micrograph of resting and activated platelets.

Platelet Agonists (Activating Agents) Used in Aggregometry

The optical PRP-based aggregation method is employed most frequently in clinical practice, and the agonists used are thrombin or synthetic *thrombin receptor-activating peptide* (TRAP), adenosine diphosphate (ADP), epinephrine, collagen, arachidonic acid, and ristocetin. Table 42-5 lists representative concentrations and platelet activation pathways tested by each agonist. Small volumes (2 to 5 μL) of concentrated agonist are

TABLE 42-4 Typical Normal Ranges in Platelet Lumiaggregometry

Agonist	Final Concentration	Aggregation Recorded as Impedance	ATP Secretion
Thrombin	1 unit/mL	Not recorded, as thrombin often causes clotting	1.0–2.0 nM
TRAP	10, 50, 100 μM	15–27 Ω	1.0–2.0 nM
Collagen	1 $\mu\text{g/mL}$	15–27 Ω	0.5–1.7 nM
	5 $\mu\text{g/mL}$	15–31 Ω	0.9–1.7 nM
ADP	5 μM	1–17 Ω	0.0–0.7 nM
	10 μM	6–24 Ω	0.4–1.7 nM
Arachidonic acid	500 μM	5–17 Ω	0.6–1.4 nM
Ristocetin	1 mg/mL	>10 Ω	Not recorded

TRAP, Thrombin receptor-activating peptide; ATP, adenosine triphosphate; ADP, adenosine diphosphate.

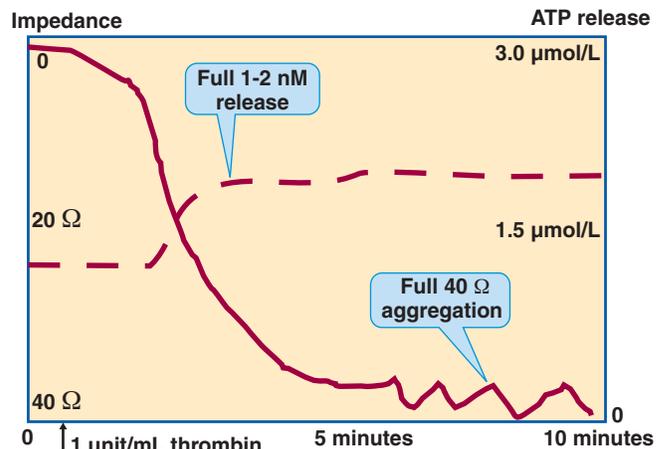


Figure 42-6 Normal lumiaggregometry tracing illustrating monophasic aggregation curve with superimposed release (secretion) reaction curve. Aggregation is measured in ohms (Ω) using the left y-axis scale; release is measured in μM of adenosine triphosphate (ATP) based on luminescence using the right y-axis scale. Curve illustrates full aggregation and secretion response to 1 unit/mL of thrombin. (Courtesy Margaret Fritsma, University of Alabama at Birmingham.)

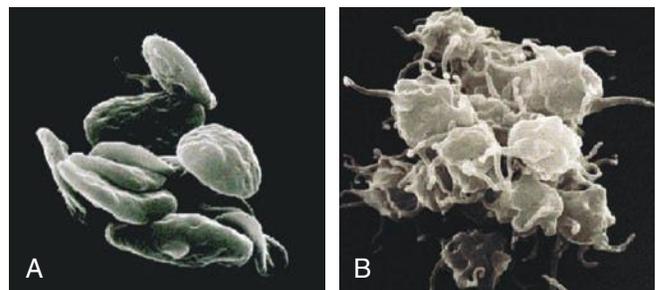


Figure 42-7 Scanning electron micrograph of resting (A) and activated (B) platelets.

TABLE 42-5 Platelet Aggregometry Agonists, Reaction Concentrations, and Platelet Receptors

Agonist	Typical Final Concentration	Platelet Membrane Receptors
Thrombin	1 unit/mL	PAR1 and PAR4; GP Ib α and GP V
ADP	1–10 μ M	P2Y ₁ , P2Y ₁₂
Epinephrine	2–10 μ g/mL	α_2 -adrenergic receptor
Collagen	5 μ g/mL	GP Ia/IIa, GP VI
Arachidonic acid	500 μ M	TP α , TP β
Ristocetin	1 mg/mL	GP Ib/IX/V in association with von Willebrand factor

GP, Glycoprotein; PAR, protease-activatable receptor; P2Y, platelet membrane ADP-receptor; TP, thromboxane receptor.

used so that they have little dilutional effect in the reaction system.³⁷

Thrombin (or TRAP) cleaves two platelet membrane protease-activatable receptors (PARs), PAR-1 and PAR-2, both members of the seven-transmembrane repeat receptor family (Chapter 13). Thrombin or TRAP also cleaves glycoprotein (GP) 1b α and GP V. Internal platelet activation is effected by membrane-associated G proteins and both the *eicosanoid* and the *diacylglycerol* pathways. Thrombin-induced activation results in full secretion and aggregation. In lumiaggregometry, the operator ordinarily begins with 1 unit/mL of thrombin or TRAP (agonist concentrations are expressed as final reaction mixture concentrations) to induce the release of 1 to 2 nM of ATP, detected by the firefly luciferin-luciferase luminescence assay. Other agonists—for instance, 5 μ g/mL of collagen—induce the release of at most 0.5 to 1.0 nM of ATP. Thrombin-induced secretion may be diminished to less than 1 nM in storage pool deficiencies (Chapter 41), but it is relatively unaffected by membrane disorders or pathway enzyme deficiencies.

Reagent thrombin is stored dry at -20° C and is reconstituted with physiologic saline immediately before use. Leftover reconstituted thrombin may be divided into aliquots, frozen, and thawed for later use. Thrombin has the disadvantage that it often triggers coagulation (fibrin formation) simultaneously with aggregation. The use of TRAP avoids this pitfall.

ADP binds platelet membrane receptors P2Y₁ and P2Y₁₂, also members of the seven-transmembrane repeat receptor family. ADP-induced platelet activation relies on the physiologic response of membrane-associated G protein and the eicosanoid synthesis pathway. The end product of eicosanoid synthesis, thromboxane A₂, raises cytosolic free calcium, which mediates platelet activation and induces secretion of ADP stored in dense granules. The secreted ADP activates neighboring platelets.

ADP is the most commonly used agonist, particularly in aggregometry systems that measure only aggregation and not luminescence. The operator adjusts the ADP concentration to between 1 and 10 μ M to induce “biphasic” aggregation (Figure 42-4). At ADP concentrations near 1 μ M, platelets achieve only *primary aggregation*, followed by disaggregation. The graph line deflects from the baseline for 1 to 2 minutes and then returns to baseline.

Primary aggregation involves shape change with formation of microaggregates, both reversible. *Secondary aggregation* is the formation of full platelet aggregates after release of platelet dense-granule ADP. At agonist ADP concentrations near 10 μ M, there is simultaneous irreversible shape change, secretion, and formation of aggregates, resulting in a monophasic curve and full deflection of the tracing. ADP concentrations between 1 and 10 μ M induce a biphasic curve: primary aggregation followed by a brief flattening of the curve called the *lag phase* and then secondary aggregation.

Operators expend considerable effort to discover the ADP concentration that generates a biphasic curve with a visible lag phase because the appropriate concentration varies among patients. This enables operators to use aggregometry alone to distinguish between membrane-associated platelet defects and storage pool or release defects.

Lumiaggregometry provides a clearer and more reproducible measure of platelet secretion, rendering the quest for the biphasic curve unnecessary. Secretion in response to ADP at 5 μ M is diminished in platelet membrane disorders; eicosanoid synthesis pathway enzyme deficiencies; or aspirin, NSAID, or clopidogrel therapy. Secretion is absent in storage pool deficiency when thrombin or TRAP is used as the agonist.

Reagent ADP is stored at -20° C, reconstituted with physiologic saline, and used immediately after reconstitution. Leftover reconstituted ADP may be aliquotted and frozen for later use.

Epinephrine binds platelet α -adrenergic receptors, identical to muscle receptors, and activates the platelets through the same metabolic pathways as reagent ADP. The results of epinephrine-induced aggregation match those of ADP, except that epinephrine cannot induce aggregation in storage pool disorder or eicosanoid synthesis pathway defects no matter how high its concentration. Epinephrine does not work in whole-blood aggregometry.

Epinephrine is stored at 1° C to 6° C and reconstituted with distilled water immediately before it is used. Leftover reconstituted epinephrine may be aliquotted and frozen for later use.

Collagen binds GP Ia/IIa and GP VI, but it induces no primary aggregation. After a lag of 30 to 60 seconds, aggregation begins, and a monophasic curve develops. Aggregation induced by collagen at 5 μ g/mL requires intact membrane receptors, functional membrane G proteins, and normal eicosanoid pathway function. Loss of collagen-induced aggregation may indicate a membrane abnormality, storage pool disorder, release defect, or the presence of aspirin.

Most laboratory managers purchase lyophilized fibrillar collagen preparations such as Chrono-Par Collagen (Chronolog Corp.). Collagen is stored at 1° C to 6° C and used without further dilution. Collagen may not be frozen.

Arachidonic acid assesses the viability of the eicosanoid synthesis pathway. Free arachidonic acid agonist at 500 μ M is added to induce a monophasic aggregometry curve with virtually no lag phase. Aggregation is independent of membrane integrity. Deficiencies in eicosanoid pathway enzymes, including deficient or aspirin-suppressed cyclooxygenase, result in reduced aggregation and secretion.

Arachidonic acid is readily oxidized and must be stored at -20° C in the dark. The operator dilutes arachidonic acid with

a solution of bovine albumin for immediate use. Aliquots of bovine albumin–dissolved arachidonic acid may be frozen for later use.

Platelet Aggregometry Tests in von Willebrand Disease

Ristocetin-Induced Platelet Aggregation. Although this test is usually called the *ristocetin-induced platelet aggregation (RIPA) test*, ristocetin actually induces an *agglutination* reaction that involves little platelet shape change and little secretion. A normal RIPA result may imply that normal concentrations of functional VWF are present and that the platelets possess a functional VWF receptor, GP Ib/IX/V (Chapter 13).³⁸

Using light transmittance aggregometry, ristocetin at 1 mg/mL final concentration induces a monophasic aggregation tracing from a normal specimen. Specimens from patients with VWD, except for subtype 2B VWD, produce a reduced or absent reaction, although all other agonists generate normal tracings (Table 38-4). Exogenous VWF from normal plasma restores a normal RIPA reaction, confirming the diagnosis (Chapter 38). In patients with Bernard-Soulier syndrome, a congenital abnormality of the GP Ib or IX portion of the GP Ib/IX/V receptor results in a diminished RIPA reaction that is not corrected by the addition of VWF (Chapter 41).

In VWD subtype 2B, a VWF gain-of-function mutation, aggregation occurs even when reduced ristocetin concentrations (down to 0.1 mg/mL final concentration) are added. This response illustrates the increased affinity of large VWF multimers for platelet receptors. The low-dose or low-concentration RIPA, sometimes called the *ristocetin response curve*, is used to diagnose type 2B VWD.

The RIPA test is qualitative and is diagnostic in only about 70% of cases. Most laboratory managers have dropped RIPA from their test menus because of its poor predictive value. There is considerable variation in laboratory results from one patient to another in the same kindred and from time-to-time in a single patient. Consequently, the laboratory director must include the *ristocetin cofactor test*, the *VWF antigen immunoassay*, and the *coagulation factor VIII activity assay* in the VWD profile. Many laboratories also offer the *VWF activity immunoassay* and the *VWF collagen-binding assay*. Ultimate confirmation and characterization of VWD is based on gel immunoelectrophoresis to characterize VWF monomers (Chapter 38).³⁹

Ristocetin Cofactor Assay for von Willebrand Factor Activity. One essential refinement of ristocetin aggregometry is the substitution of formalin-fixed or lyophilized normal “reagent” platelets for the patient’s platelets.⁴⁰ When reagent platelets are used, the test is called the *ristocetin cofactor* or *VWF activity assay*. The medical laboratory practitioner prepares the patient’s PPP; mixes it with reagent platelets; adds ristocetin; and performs optical, not impedance, aggregometry. The ristocetin cofactor assay yields a proportional relationship between VWF activity and the aggregometry response of the reagent platelets. Comparison of the aggregation results for patients’ PPP with those for standard dilutions of normal “reagent” PPP permits a quantitative expression of the VWF activity level. The

ristocetin cofactor test also is available as an automated assay on the BCT coagulometer and the BCS coagulometer (Siemens Healthcare Diagnostics, Deerfield, IL), which use latex particles in place of preserved platelets.

VWF Activity Immunoassay and VWF Activity Collagen Binding Assay. Although the ristocetin cofactor assay has been used for many years to measure VWF activity, it offers consistently poor precision, as illustrated by external quality assurance survey results.⁴¹ Two additional assays, the VWF Activity Immunoassay (for instance, the REAADS von Willebrand Factor Activity enzyme immunoassay, DiaPharma, West Chester, OH) and the VWF Collagen Binding Assay (Technozym vWF:CBA ELISA Collagen Type I, DiaPharma, West Chester, OH) are available. The former employs a monoclonal antibody specific for an active VWF epitope, and the latter mimics VWF’s in vivo collagen adhesion property. Both reflect VWF activity rather than concentration and offer improved precision when compared to the ristocetin cofactor assay.

Summary of Lumiaggregometry Agonist Responses in Various Circumstances

Thrombin produces maximum ATP release through at least two membrane-binding sites. Laboratory practitioners use collagen, ADP, and epinephrine to test for abnormalities of their respective membrane binding sites and the eicosanoid synthesis pathway. Arachidonic acid is the agonist that practitioners use to check for eicosanoid synthesis deficiencies. Ristocetin is used to check for abnormalities of plasma VWF in VWD. The following conditions may be detected through platelet lumiaggregometry.

Therapy with Aspirin, Other Nonsteroidal Anti-Inflammatory Drugs, and Clopidogrel. NSAIDs such as aspirin, ibuprofen, indomethacin, and sulfapyrazone permanently inactivate or temporarily inhibit cyclooxygenase. The thienopyridine antiplatelet drugs *clopidogrel* and *prasugrel* irreversibly occupy the ADP receptor P2Y₁₂ whereas the *nucleoside ticagrelor* is reversibly bound (Chapter 41). The NSAIDs limit or eliminate the aggregation and secretion responses to arachidonic acid and collagen. The P2Y₁₂ inhibitors suppress aggregation and secretion responses to ADP.⁴² Platelet aggregometry is employed to monitor response to these antiplatelet drugs (Chapter 41).⁴³ The VerifyNow system (Accumetrics, San Diego, CA) with specific assays for monitoring the effect of aspirin, the P2Y₁₂ inhibitors, and the GP IIb/IIIa inhibitor antiplatelet drugs is gaining favor in point-of-care settings (Chapter 44). The physician or medical laboratory practitioner must instruct the patient to discontinue all antiplatelet drugs at least 1 week before blood is collected for aggregometry unless aggregometry is ordered to monitor the effects of these drugs.

Platelet Release (Secretion) Defects: Eicosanoid Pathway Enzyme Deficiencies. Congenital or acquired deficiencies of cyclooxygenase, thromboxane synthase, protein kinase C, or any enzyme in the eicosanoid activation pathway limit or

prevent secretion. Thrombin may induce normal responses, but secretion and aggregation are diminished in response to ADP, collagen, and arachidonic acid. Because the aggregation responses resemble the responses seen during the use of NSAIDs, release defects are often called *aspirin-like disorders*.

Storage Pool Deficiency. In a congenital or acquired storage pool defect, dense granules are empty or missing. ATP release in response to thrombin is reduced, as it is in response to ADP, arachidonic acid, and collagen (Table 42-6).

Platelet Membrane Defects: Thrombasthenia. Glanzmann thrombasthenia, a membrane defect characterized by dysfunction or loss of the GP IIb/IIIa receptor site, may be diagnosed by its characteristically diminished secretion and aggregation responses to all agonists except thrombin or its modest response to arachidonic acid.

Acquired Platelet Disorders. Platelets may become either dysfunctional or hyperactive in acquired hematologic and systemic disorders such as acute leukemia, aplastic anemia, myeloproliferative neoplasms, myelodysplastic syndromes, myeloma, uremia, liver disease, and chronic alcohol abuse. The physician looks for these disorders in any case where aggregation is abnormal and no other explanation is available. Platelet aggregometry results may predict the risk of bleeding or thrombosis in the patient with acquired platelet function disorders.⁴⁴

Testing for Heparin-Induced Thrombocytopenia

A description of the clinical manifestations and mechanism of heparin-induced thrombocytopenia (HIT) is provided in Chapter 39, and a summary of laboratory tests for HIT is provided in Chapter 40. Aggregation tests for HIT include light-transmittance aggregometry, washed platelet light-transmittance aggregometry, washed platelet lumiaggregometry, and whole-blood lumiaggregometry.⁴⁵⁻⁴⁷ The washed platelet carbon-14 (¹⁴C) serotonin release assay (SRA) is based on platelet activation and secretion. All these tests employ unfractionated heparin as their agonist. The ¹⁴C-SRA is available from specialized reference laboratories and is regarded as the reference confirmatory method. Few local institutions

provide the ¹⁴C-SRA, because a radionuclide license is required. Except for the ¹⁴C-SRA, aggregometry and lumiaggregometry tests for HIT have proven to be insensitive and have been largely discontinued.

QUANTITATIVE MEASUREMENT OF PLATELET MARKERS

Immunoassay for the Anti-Platelet Factor 4 (Heparin-Induced Thrombocytopenia) Antibody

Amiral and colleagues developed a HIT screening immunoassay based on their discovery that PF4 is the target for the heparin-dependent antiplatelet antibody that causes HIT (Chapter 40).⁴⁸ One adaptation of this principle is the PF4 Enhanced Solid Phase ELISA (Hologic Gen-Probe Inc., San Diego, CA). Patient plasma is incubated in microtiter plate wells that are coated with a solid-phase complex of purified PF4 and polysulfonate, a plastic molecule integral to plate construction that resembles heparin. Heparin-dependent anti-PF4 antibodies bind the PF4-polysulfonate complex. Bound antibodies are detected using enzyme-conjugated anti-human immunoglobulin G (IgG), IgA, and IgM antibodies and a substrate chromophore. This test is more sensitive than the ¹⁴C-SRA and detects antibodies early in the development of HIT, but it may detect antibodies that are unaccompanied by clinical symptoms, known as *biologic false positives*.⁴⁹ A second kit that uses only enzyme-conjugated anti-human IgG is more specific. Other immunoassay kits employ PF4-heparin in place of PF4-polysulfonate and have similar sensitivity and specificity characteristics.

Assays for Platelet Activation Markers

Elevated plasma levels of the platelet-specific proteins β -thromboglobulin and PF4 may accompany thrombotic stroke or coronary thrombosis.⁵⁰ The implication that in vivo platelet activation contributes to the condition or that the measurement of these proteins is of diagnostic or prognostic significance is under investigation.⁵¹ Diagnostica Stago, Inc. (Parsippany, NJ) produces enzyme immunoassay kits for PF4 and β -thromboglobulin (β -TG) under the brand names Asserachrom PF4 and Asserachrom β -TG. Special collection techniques are necessary because PF4 and β -TG test results may be invalidated by platelet activation during and even subsequent to specimen collection.⁵² CTAD tubes (refer to the section on Hemostasis Specimen Collection above) are required for specimen collection for PF4 and β -TG assays. Plasma must undergo extraction before PF4 and β -TG assays are performed because several eicosanoids cross-react with kit antibodies and falsely raise the results.

Thromboxane A₂, the active product of the eicosanoid pathway, has a half-life of 30 seconds, diffuses from the platelet, and spontaneously reduces to thromboxane B₂, a stable, measurable plasma metabolite (Chapter 13). Efforts to produce a clinical assay for plasma thromboxane B₂ have been unsuccessful because specimens must be collected in CTAD tubes to prevent in vitro platelet activation and must undergo

TABLE 42-6 Expected Platelet Lumiaggregometry Results in Storage Pool Disorder for a Variety of Agonists

Agonist	Final Concentration	Aggregation Recorded as Impedance	ATP Secretion
Thrombin	1 unit/mL	Not recorded*	<0.1 nM
TRAP	10, 50, 100 μ M	20 Ω	<0.1 nM
Collagen	5 μ g/mL	20 Ω	<0.1 nM
Arachidonic acid	500 μ M	12 Ω	<0.1 nM

*Thrombin aggregation is not recorded as thrombin typically causes clotting, which interferes with the aggregation response. TRAP avoids the clotting response. ATP, Adenosine triphosphate.

an extraction step before the assay is performed. Thromboxane B₂ is acted on by liver enzymes to produce an array of soluble urine metabolites, including 11-dehydrothromboxane B₂, which is stable and measurable.⁵³ Immunoassays of urine 11-dehydrothromboxane B₂ are employed to characterize *in vivo* platelet activation.⁵⁴ These assays require no special specimen management and can be performed on random urine specimens. The urinary 11-dehydrothromboxane B₂ assay also may be used to monitor aspirin therapy and to identify cases of therapy failure or aspirin resistance.⁵⁵

CLOT-BASED PLASMA PROCOAGULANT SCREENS

The Lee-White whole-blood coagulation time test, described in 1913, was the first laboratory procedure designed to assess coagulation.⁵⁶ The Lee-White test is no longer used, but it was the first *in vitro* clot procedure that employed the principle that the time interval from the initiation of clotting to visible clot formation reflects the condition of the coagulation mechanism. A prolonged clotting time indicates a coagulopathy (coagulation deficiency). A 1953 modification, the *activated clotting time* (ACT) test, utilizes a particulate clot activator in the test tube, which speeds the clotting process. The ACT is still widely used as a point-of-care assay to monitor heparin therapy in high-dosage applications such as percutaneous intervention (cardiac catheterization) and coronary artery bypass graft surgery (Chapter 43).

The standard clot-based coagulation screening tests—PT, PTT, fibrinogen assay, and thrombin clotting time (TCT)—use the clotting time principle of the Lee-White test. Many specialized tests, such as coagulation factor assays, tests of fibrinolysis, inhibitor assays, reptilase time, Russell viper venom time, and dilute Russell viper venom time, are also based on the relationship between time to clot formation and coagulation function.

Prothrombin Time

Prothrombin Time Principle

PT reagents, often called *thromboplastin* or *tissue thromboplastin*, are prepared from recombinant or affinity-purified *tissue factor* suspended in phospholipids mixed with a buffered 0.025 M solution of calcium chloride.⁵⁷ A few less responsive thromboplastins are organic extracts of emulsified rabbit brain or lung suspended in calcium chloride. When mixed with citrated PPP, the PT reagent triggers fibrin polymerization by activating plasma factor VII (Figure 42-8). Calcium and phospholipids participate in the formation of the tissue factor–factor VIIa complex, the factor VIIIa–factor IXa complex, and the factor Va–factor Xa complex. The clot is detectable visually or by optical or electromechanical sensors. Although the coagulation pathway implies that the PT would be prolonged in deficiencies of fibrinogen, prothrombin, and factors V, VII, VIII, IX, and X, the procedure is most sensitive to factor VII deficiencies, moderately sensitive to factor V and X deficiencies, sensitive to severe fibrinogen and prothrombin deficiencies, and insensitive to deficiencies of factors VIII and IX.^{58,59} The PT is prolonged in multiple factor deficiencies that include deficiencies of factors VII and X and is used most often to monitor the effects of therapy with the oral anticoagulant Coumadin (Chapter 43).

Prothrombin Time Procedure

The tissue factor-phospholipid-calcium chloride reagent is warmed to 37° C. An aliquot of test PPP, 50 or 100 μL, is transferred to the reaction vessel, which also is maintained at 37° C. The PPP aliquot is incubated at 37° C for at least 3 and for no more than 10 minutes. Aliquots that are incubated longer than 10 minutes become prolonged as coagulation factors begin to deteriorate or are affected by evaporation and pH change. A premeasured volume of reagent, 100 or 200 μL, is directly and quickly added to the PPP aliquot, and a timer is started. As the

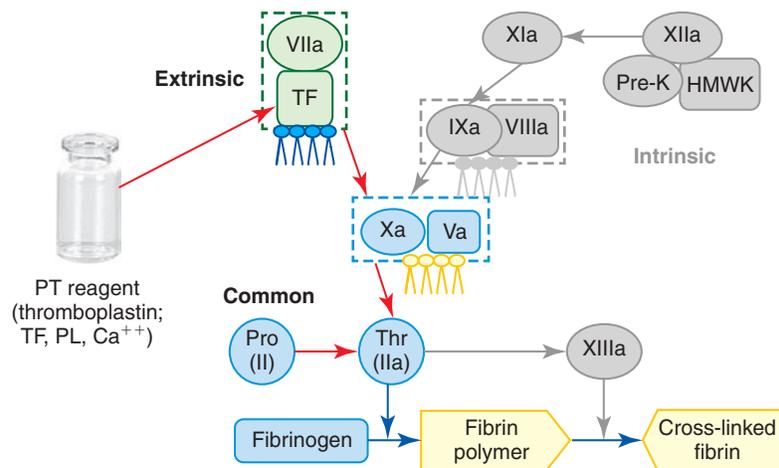


Figure 42-8 Prothrombin time (PT) reagent (thromboplastin) consists of tissue factor (TF), phospholipid (PL), and ionized calcium (Ca⁺⁺). The reagent activates the extrinsic and common pathways of the coagulation mechanism beginning with factor VII (see colored area in figure). The PT is prolonged by deficiencies of factors VII, X, and V; prothrombin; and fibrinogen when the fibrinogen level is less than 100 mg/dL. The PT is prolonged in Coumadin therapy because Coumadin suppresses production of factor VII, factor X, and prothrombin. Factor VII has a 6-hour half-life and has the earliest effect on the PT. The PT does not detect factor XIII deficiency. *HMWK*, High-molecular-weight kininogen (Fitzgerald factor); *Pre-K*, prekallikrein (Fletcher factor); *Pro*, prothrombin (II, zymogen); *Thr*, thrombin (activated factor II, or IIa; serine protease); *Va*, *VIIIa*, activated factors V and VIII (serine protease cofactors); *VIIa*, *IXa*, *Xa*, *XIa*, activated factors VII, IX, X, XI (serine proteases); *XIIa*, activated factor XII (serine protease, but not part of *in vivo* coagulation); *XIIIa*, activated factor XIII (transglutaminase).

clot forms, the timer stops, and the elapsed time is recorded. If the procedure is performed in duplicate, the duplicate values must be within 10% of their mean or the test is repeated for a third time. Most laboratory practitioners perform PTs using automated instruments that strictly control temperature, pipetting, and interval timing (Chapter 44). With automated instruments, duplicate testing is unnecessary.

Prothrombin Time Quality Control

The medical laboratory practitioner tests normal and prolonged control PPP specimens at the beginning of each 8-hour shift or with each change of reagent. Although lyophilized control PPPs are commercially available, the laboratory manager may choose to collect and pool PPP specimens from designated subjects to make “laboratory-developed” controls. In this case, the specimens must be collected and managed using the same tubes, anticoagulant, and protocol that are used for patient plasma specimen collection. The samples are pooled, tested, and aliquotted. Regardless of whether commercial or locally prepared controls are used, the control is tested alongside patient specimens using the same protocol as for patient PPP testing.

The normal control result should be within the reference interval, and the prolonged control result should be within the therapeutic range for Coumadin. If the control results fall within the stated limits provided in the laboratory protocol, the test results are considered valid. If the results fall outside the control limits, the reagents, control, and equipment are checked; the problem is corrected; and the control and patient specimens are retested. The operator records all the actions taken. Control results are recorded and analyzed at regular intervals to determine the long-term validity of results.

Reporting of Prothrombin Time Results and the International Normalized Ratio

The medical laboratory practitioner reports PT results to the nearest tenth of a second, along with the PT reference interval. If the PT assay is performed in duplicate, the results are averaged, and the average is reported.

For Coumadin monitoring, to compensate for the inherent variations among thromboplastin reagents, most laboratories report the *international normalized ratio* (INR) for patients with a stable anticoagulation response using the following formula:⁶⁰

$$\text{INR} = (PT_{\text{patient}}/PT_{\text{geometric mean of normal}})^{\text{ISI}}$$

where PT_{patient} is the PT of the patient in seconds, $PT_{\text{geometric mean of normal}}$ is the PT of the geometric mean of the reference interval, and *ISI* is the *international sensitivity index*. Reagent producers generate the ISI for their thromboplastin by performing an orthogonal regression analysis comparing its PT results on a set of plasmas, with the results obtained using the international reference thromboplastin preparation (World Health Organization human brain thromboplastin). Most responsive thromboplastin reagents have ISIs near 1, the assigned ISI of the

WHO reagent. Automated coagulation instruments “request” the reagent ISI from the operator or incorporate it from the reagent label bar code and compute the INR for each specimen. INRs are meant to be computed only for samples from patients who have achieved a stable anticoagulation response with Coumadin. During the first week of Coumadin therapy, the physician should interpret PT results in seconds, comparing them with the reference interval. Chapter 43 provides a full discussion of Coumadin therapy monitoring.

Localized ISI calibration is replacing reagent manufacturer-generated ISIs as it produces a laboratory-specific ISI value that is likely to be more accurate than a distributor-provided ISI.⁶¹ The laboratory practitioner performs PTs on a set of four to five calibrator plasmas—for instance, ISI Calibrate (Instrumentation Laboratory, Bedford, MA). The calibrators arrive with predetermined PT values. If calibrators are not available, the practitioner may use a series of 100 patient specimens. The practitioner prepares a linear graph with the preestablished calibrator PTs or the PT values of the 100 patient specimens using the lab’s current PT reagent on the Y scale and local PTs using the new reagent on the X scale and computes the slope. The reference ISI provided by the manufacturer for the new PT reagent is multiplied by the slope value to produce the local ISI of the new PT reagent.

The same approach may be applied to lot-to-lot calibrations of PT reagents; however, in most lot-to-lot validations the operator need only assay a three-level validation plasma set—for instance, ISI Validate (Instrumentation Laboratory, Bedford, MA). If the lot values determined using the new reagent are within predetermined limits, the lot may be placed in everyday operation without a change; if not, it is necessary to recalibrate the ISI value of the new lot of the PT reagent.

Prothrombin Time Reference Interval

The PT reference interval, computed from PT values of healthy individuals, varies from site to site, depending on the patient population, type of thromboplastin used, type of instrument used, and pH and purity of the reagent diluent. Each center must establish its own range for each new lot of reagents, or at least once a year. This may be done by testing a sample of at least 30 specimens from healthy donors of both sexes spanning the adult age range over several days and computing the 95% confidence interval of the results. A typical PT reference interval is 12.6 to 14.6 seconds.

The Prothrombin Time as a Diagnostic Assay

The PT is performed diagnostically when any coagulopathy is suspected. Acquired multiple deficiencies such as disseminated intravascular coagulation (DIC), liver disease, and vitamin K deficiency all affect factor VII activity and are detected through prolonged PT results. The PT is particularly sensitive to liver disease, which causes factor VII levels to become rapidly diminished (Chapter 38).

Vitamin K deficiency is seen in severe malnutrition, during use of broad-spectrum antibiotics that destroy gut flora, with parenteral nutrition, and in malabsorption syndromes. Vitamin K levels

are low in newborns, in which bacterial colonization of the gut has not begun. Hemorrhage is likely in vitamin K deficiency, and the PT is the best indicator. To distinguish between vitamin K deficiency and liver disease, the laboratory practitioner determines factor V and factor VII levels. Both factor V and factor VII are reduced in liver disease; only factor VII is reduced in vitamin K deficiency. Chapter 38 provides details regarding liver disease and vitamin K deficiency.

The PT is prolonged in congenital single-factor deficiencies of factor X, VII, or V; profound prothrombin deficiency; and fibrinogen deficiency when the fibrinogen level is 100 mg/dL or less. When the PT is prolonged but the PTT and thrombin clotting time (TCT) test results are normal, factor VII activity may be deficient. Any suspected single-factor deficiency is confirmed with a factor assay. The PT is not affected by factor VIII or IX deficiency, because the concentration of tissue factor in the reagent is high, and those factors are bypassed in thrombin generation.

Minimal Effectiveness of Prothrombin Time as a Screening Tool

Preoperative PT screening of asymptomatic surgical patients to predict intraoperative hemorrhage is not supported by prevalence studies, unless the patient is a member of a high-risk population.^{62,63} No clinical data support the use of the PT as a general screening test for individuals at low risk of bleeding, and the PT is not useful for establishing baseline values in Coumadin therapy.⁶⁴ The therapeutic target range for Coumadin therapy is based on the INR, not the baseline PT result or PT control value.

Limitations of the Prothrombin Time

Specimen variations profoundly affect PT results (Table 42-7). The ratio of whole blood to anticoagulant is crucial, so collection tubes must be filled to within tube manufacturers' specifications

TABLE 42-7 Factors That Interfere with the Validity of Clot-based Test Results

Problem	Solution
Blood collection volume less than specified minimum	PT falsely prolonged; recollect specimen.
Hematocrit $\geq 55\%$	Adjust anticoagulant volume using formula and recollect specimen using new anticoagulant volume.
Clot in specimen	All results are affected unpredictably; recollect specimen.
Visible hemolysis	PT falsely shortened; recollect specimen.
Icterus or lipemia	Measure PT using a mechanical coagulometer.
Heparin therapy	Use reagent known to be insensitive to heparin or one that includes a heparin neutralizer such as polybrene.
Lupus anticoagulant	PT result is invalid; use chromogenic factor X assay instead of PT.
Incorrect calibration, incorrect dilution of reagents	Correct analytical error and repeat test.

and not underfilled or overfilled. Anticoagulant volume must be adjusted when the hematocrit is greater than 55% to avoid false prolongation of the results. Specimens must be inverted five times immediately after collection to ensure good anticoagulation, but the mixing must be gentle. Practitioners must reject clotted and visibly hemolyzed specimens because they give unreliable results. Plasma lipemia or icterus may affect the results obtained with optical instrumentation.

Heparin may prolong the PT. If the patient is receiving therapeutic heparin, it should be noted on the order and commented on when the results are reported. The laboratory manager selects thromboplastin reagents that are maximally sensitive to oral anticoagulant therapy and insensitive to heparin. Many reagent manufacturers incorporate polybrene (5-dimethyl-1,5-diazaundecamethylene polymethobromide, hexadimethrine bromide, Sigma-Aldrich, St. Louis, MO) in their thromboplastin reagent to neutralize heparin. The medical laboratory practitioner may detect unexpected heparin by using the TCT test, which is described subsequently.

Lupus anticoagulants (LAs) prolong some thromboplastins. LAs are members of the antiphospholipid antibody family and may partially neutralize PT reagent phospholipids. Coumadin often is prescribed to prevent thrombosis in patients with LAs, but the PT may be an unreliable monitor of therapy in such cases. Patients who have an LA and are taking Coumadin should be monitored using an alternative system, such as the chromogenic factor X assay.^{65,66}

Reagents must be reconstituted with the correct diluents and volumes following manufacturer instructions. Reagents must be stored and shipped according to manufacturer instructions and never used after the expiration date.

Partial Thromboplastin Time

Partial Thromboplastin Time Principle

The PTT (also called the *activated partial thromboplastin time*, or APTT) is performed to monitor the effects of unfractionated heparin therapy and to detect LA and specific anticoagulation factor antibodies such as anti-factor VIII antibody. The PTT is also prolonged in all congenital and acquired procoagulant deficiencies, except for deficiencies of factor VII or XIII.⁶⁷

The PTT reagent contains phospholipid (previously called *partial thromboplastin*) and a negatively charged particulate activator such as silica, kaolin, ellagic acid, or celite in suspension. The phospholipid mixture, which was historically extracted from rabbit brain, is now produced synthetically. The activator provides a surface that mediates a conformational change in plasma factor XII that results in its activation (Figure 42-9). Factor XIIa forms a complex with two other plasma components: high-molecular-weight kininogen (*Fitzgerald factor*) and prekallikrein (*Fletcher factor*). These three plasma glycoproteins, termed the *contact activation factors*, initiate in vitro clot formation through the *intrinsic* pathway but are not part of in vivo coagulation. Factor XIIa, a serine protease, activates factor XI (XIa), which activates factor IX (IXa) (Chapter 37).

Factor IXa binds calcium, phospholipid, and factor VIIIa to form a complex. In the PTT reaction system, ionic calcium and

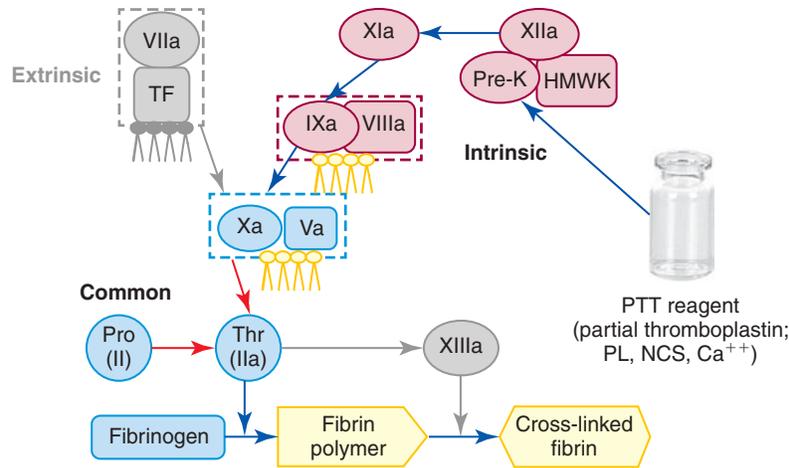


Figure 42-9 Partial thromboplastin time (PTT) reagent (partial thromboplastin) consists of phospholipid (PL), a negatively charged particulate activator (NCS), and ionized calcium. It activates the intrinsic and common pathways of the coagulation mechanism through the contact factors XII, prekallikrein (Pre-K; also called *Fletcher factor*), and high-molecular-weight kininogen (HMWK; also called *Fitzgerald factor*), none of which is significant in the *in vivo* coagulation mechanism (see colored area in figure). The PTT is prolonged by deficiencies in Pre-K; HMWK; factors XII, XI, IX, VIII, X, and prothrombin; and fibrinogen when the fibrinogen level is less than 100 mg/dL. The deficiencies for which the PTT reagent is specifically calibrated are factors VIII, IX, and XI. The PTT is prolonged in heparin therapy because heparin activates plasma antithrombin, which neutralizes all the plasma serine proteases, particularly thrombin (IIa) and activated factor X (Xa). The PTT is prolonged in the presence of lupus anticoagulant because the anticoagulant neutralizes essential reagent phospholipids. The PTT does not detect factor XIII deficiency. *TF*, Tissue factor; *Pro*, prothrombin (II, zymogen); *Thr*, thrombin (activated factor II, or IIa; serine protease); *Va*, *VIIIa*, activated factors V and VIII (serine protease cofactors); *VIIa*, *IXa*, *Xa*, *XIa*, activated factors VII, IX, X, XI (serine proteases); *XIIa*, activated factor XII (serine protease, but not part of *in vivo* coagulation); *XIIIa*, activated factor XIII (transglutaminase).

phospholipid are supplied in the reagent. The factor IXa–calcium–factor VIIIa–phospholipid complex catalyzes factor X (Xa). Factor Xa forms another complex with calcium, phospholipid, and factor Va, catalyzing the conversion of prothrombin to thrombin. Thrombin catalyzes the polymerization of fibrinogen and the formation of the fibrin clot, which is the endpoint of the PTT.

The factors whose deficiencies are associated with hemorrhage and are reflected in prolonged PTT results, taken in the order of reaction, are XI, IX, VIII, X, and V; prothrombin; and fibrinogen, when fibrinogen is 100 mg/dL or less. Most PTT reagents are designed so that the PTT is prolonged when the test PPP has less than approximately 0.3 units/mL (30% of normal) of VIII, IX, or XI.⁶⁸ The PTT also is prolonged in the presence of LA, an immunoglobulin with affinity for phospholipid-bound proteins, and is prolonged by anti-factor VIII antibody, antibodies to factor IX and other coagulation factors, and therapeutic heparin. Factor VII and factor XIII deficiencies have no effect on the PTT. Deficiencies of factor XII, prekallikrein, or high-molecular-weight kininogen prolong the PTT but do not cause bleeding.

Partial Thromboplastin Time Procedure

To initiate contact activation, 50 or 100 μL of warmed (37°C) reagent consisting of phospholipid and particulate activator is mixed with an equal volume of warmed PPP. The mixture is allowed to incubate for the exact manufacturer-specified time, usually 3 minutes. Next, 50 or 100 μL of warmed 0.025 M calcium chloride is forcibly added to the mixture, and a timer is started. When a fibrin clot forms, the timer stops, and the interval is recorded. Timing may be done with a stopwatch

or by an automatic electromechanical or photo-optical device. If the PTT is performed manually, the test should be done in duplicate, and the two results must match within 10%.

Partial Thromboplastin Time Quality Control

The medical laboratory practitioner tests normal and prolonged control plasma specimens at the beginning of each 8-hour shift or with each new batch of reagent. The laboratory director may require more frequent use of controls. Controls are tested using the protocol for patient plasma testing.

The normal control result should be within the reference interval, and the abnormal control result should be within the therapeutic range for unfractionated heparin (Chapter 43). If the control results fall within the stated limits in the laboratory protocol, the test results are considered valid. If the results fall outside the control limits, the reagents, control, and equipment are checked; the problem is corrected; and the control and patient specimens are retested. The operator records each control run and all the actions taken. Control results are recorded and analyzed at regular intervals to determine the long-term validity of results.

Reagents must be reconstituted with the correct diluents and volumes following manufacturer instructions. Reagents must be stored and shipped according to manufacturer instructions and never used after the expiration date.

Specimen errors that affect the PT similarly affect the PTT (Table 42-7).

Partial Thromboplastin Time Reference Interval

The PTT reference interval varies from site to site, depending on the patient population, type of reagent, type of instrument, and

pH and purity of the diluent. One medical center laboratory has established 26 to 38 seconds as its reference interval. This range is typical, but each center must establish its own interval for each new lot of reagent, or at least once a year. This may be done by testing a sample of 30 or more specimens from healthy donors of both sexes spanning the adult age range over several days and computing the 95% confidence interval of the results.

Monitoring of Heparin Therapy with Partial Thromboplastin Time

Since the early 1970s, the PTT has been the standard method for monitoring unfractionated heparin therapy, which is used to treat patients with venous thrombosis, pulmonary embolism, myocardial infarction, and several other medical conditions.⁶⁹ The laboratory practitioner establishes a PTT therapeutic range and publishes it to all inpatient units. A typical therapeutic range is 60 to 100 seconds; however, the range varies widely and must be established locally.⁷⁰ The range must be reestablished with each change of PTT reagent, including each lot change, and upon instrument recalibration. Details on monitoring of heparin therapy and establishment of the PTT therapeutic range are provided in Chapter 43.

The Partial Thromboplastin Time as a Diagnostic Assay

The physician orders a PTT assay when a hemorrhagic disorder is suspected or when recurrent thrombosis or the presence of an autoimmune disorder points to the possibility of an LA.⁷¹ The PTT result is prolonged when there is a deficiency of one or more of the following coagulation factors: prothrombin; factor V, VIII, IX, X, XI, or XII; or fibrinogen when the fibrinogen level is 100 mg/dL or less. The PTT also is prolonged in the presence of a specific inhibitor, such as anti-factor VIII or anti-factor IX; a non-specific inhibitor, such as LA; and interfering substances, such as fibrin degradation products (FDPs) or paraproteins, which are present in myeloma.

DIC prolongs PTT results because of consumption of procoagulants, but the PTT results alone are not definitive for the diagnosis of DIC. Vitamin K deficiency results in diminished levels of procoagulant factors II (prothrombin), VII, IX, and X, and the PTT is eventually prolonged. Because factor VII deficiency does not affect the PTT, however, and because it is the first coagulation factor to become deficient, the PTT is not as sensitive to vitamin K deficiency or Coumadin therapy as the PT. The PTT is not prolonged in deficiencies of factor VII or XIII. No clinical data support the use of the PTT as a general screening test for individuals at low risk of bleeding.⁷²

Partial Thromboplastin Time Mixing Studies Lupus Anticoagulants

LAs are IgG immunoglobulins directed against a number of phospholipid-protein complexes.⁷² LAs prolong the phospholipid-dependent PTT reaction. Most laboratories employ a moderate-phospholipid or high-phospholipid PTT reagent in their primary PTT assay to monitor heparin therapy and detect coagulopathies. Laboratories use a second low-phospholipid

PTT reagent such as PTT-LA (Diagnostica Stago, Parsippany, NJ), which is more sensitive to LA, as their LA screen (Chapter 39). Because they have a variety of target antigens, LAs are called *nonspecific inhibitors*. Chronic presence of LAs confers a 30% risk of arterial or venous thrombosis; every acute care laboratory must provide a means for their detection. Together, chronic and transient LAs are found in 1% to 2% of randomly selected individuals.

Specific Factor Inhibitors

Specific factor inhibitors are IgG immunoglobulins directed against coagulation factors. Specific inhibitors arise in severe congenital factor deficiencies during factor concentrate treatment. Anti-factor VIII, the most common of the specific inhibitors, is detected in 10% to 20% of patients with severe hemophilia, and anti-factor IX is detected in 1% to 3% of factor IX-deficient patients. Autoantibodies to factor VIII occasionally may arise in individuals without hemophilia, usually in young women, where they are associated with a postpartum bleeding syndrome or in patients over 60 with autoimmune disorders. The presence of these types of antibodies is called *acquired hemophilia* (Chapter 38). Alloantibodies and autoantibodies to factor VIII are associated with severe anatomic hemorrhage.

Detection and Identification of Lupus Anticoagulants and Specific Inhibitors

LA testing is part of every thrombophilia profile (Chapter 39). An unexpectedly prolonged screening PTT may also trigger an LA investigation. PTT mixing studies are necessary for the initial detection of LAs.⁷³ Mixing studies also distinguish LAs from specific inhibitors and factor deficiencies and should be available in all coagulation laboratories.⁷⁴

When the initial PTT is prolonged beyond the upper limit of the reference interval, the laboratory practitioner first determines if heparin is present by performing the TCT. A TCT result that exceeds the upper limit of the TCT reference interval is evidence for the presence of heparin. In fact, heparin often prolongs the TCT to 30 to 40 seconds. Heparin may be neutralized using polybrene or heparinase (Hepzyme; Siemens Healthcare Diagnostics, Tarrytown, NY), and the treated sample may be used for PTT mixing studies.

The heparin-free or heparin-neutralized patient plasma is then mixed 1:1 with *reagent platelet-poor normal plasma* (PNP; Figure 39-1). Several manufacturers make PNP—for example, frozen *Cryocheck* Normal Reference Plasma (Precision BioLogic, Inc, Dartmouth, Nova Scotia). A new PTT is performed immediately on the 1:1 mixture. If the mixture PTT corrects to within 10% of the PNP PTT (or to within the reference interval) and the patient is experiencing bleeding, a coagulation factor deficiency (coagulopathy) is presumed.⁷⁵

Some LAs are time dependent and temperature dependent. Most anti-factor VIII inhibitors are temperature-dependent IgG4-class antibodies. If the immediate PTT corrects, a new mixture is prepared and incubated 1 to 2 hours at 37° C. If the incubated mixture's PTT fails to correct to within 10% of the incubated PNP PTT, an inhibitor may be

present. If the patient is bleeding, a specific inhibitor such as anti-factor VIII is suspected, and a factor VIII activity assay is performed. Although anti-factor IX and other inhibitors have been documented, anti-factor VIII is the most common. The Bethesda titer procedure, discussed later in this chapter, is used to confirm the presence of specific anti-coagulation factor antibodies.

If the PTT of the initial or incubated mixture fails to correct and the patient is not bleeding, the laboratory practitioner suspects LA and automatically orders an LA profile, as described in Chapter 39. LA profiles are available from tertiary care facilities and specialty reference laboratories.

Thrombin Clotting Time

Thrombin Clotting Time Reagent and Principle

Commercially prepared bovine thrombin reagent at 5 National Institutes of Health (NIH) units/mL cleaves fibrinopeptides A and B from plasma fibrinogen to form a detectable fibrin polymer (Figure 42-10).

Thrombin Clotting Time Procedure

Reagent thrombin is warmed to 37° C for a minimum of 3 and a maximum of 10 minutes. Thrombin deteriorates during incubation and must be used within 10 minutes of the time incubation is begun. An aliquot of PPP, usually 100 μ L, is also incubated at 37° C for a minimum of 3 and a maximum of 10 minutes. The operator pipettes 200 μ L of thrombin into the PPP aliquot, starts a timer, and records the interval to clot formation. TCT tests may be performed in duplicate and the results averaged.

Thrombin Clotting Time Quality Control

The medical laboratory practitioner tests a normal control sample and an abnormal control sample with each batch of

TCT assays and records the results. The normal control results should fall within the laboratory's reference interval. The abnormal control results should be prolonged to the range reached by the TCT in moderate hypofibrinogenemia. If the results fall outside the laboratory protocol's control limits, the reagents, control, and equipment are checked; the problem is corrected; and the control is retested. The actions taken to correct out-of-limit tests are recorded. Control results are analyzed at regular intervals (weekly is typical) to determine the longitudinal validity of the procedure.

Specimen errors that affect the PT likewise affect the TCT (Table 42-7).

Reporting of Thrombin Clotting Time Results and Clinical Utility

A typical TCT reference interval is 15 to 20 seconds, although the reference interval should be established locally. The TCT is prolonged when the fibrinogen level is less than 100 mg/dL (hypofibrinogenemia) or in the presence of antithrombotic materials such as FDPs, paraproteins, or heparin. Afibrinogenemia (absence of fibrinogen) and dysfibrinogenemia (presence of fibrinogen that is biochemically abnormal and nonfunctional) also cause a prolonged TCT. Before a prolonged TCT may be considered as evidence of diminished or abnormal fibrinogen, the presence of antithrombotic substances, such as heparin, FDPs, or paraproteins, must be ruled out. The TCT is part of the PTT mixing study protocol and is used to determine whether heparin is present whenever the PTT is prolonged.⁷⁶

The TCT may also assess the presence of the oral direct thrombin inhibitor dabigatran. The TCT provides binary (qualitative) evidence for dabigatran; if drug is present, the TCT is markedly prolonged. A normal TCT rules out dabigatran.

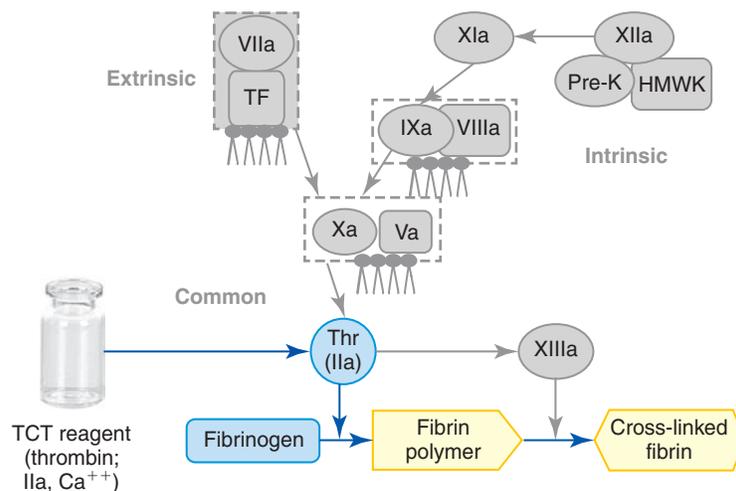


Figure 42-10 Thrombin clotting time (TCT, also reptilase time) coagulation pathway. The reagent activates the coagulation pathway at the level of thrombin and tests for the polymerization of fibrinogen (see colored area in figure). The TCT is prolonged by unfractionated heparin; direct thrombin inhibitors; fibrin degradation products; M-proteins; and dysfibrinogenemia, hypofibrinogenemia, and afibrinogenemia. The reptilase time is unaffected by heparin but is prolonged by dysfibrinogenemia, hypofibrinogenemia, and afibrinogenemia. Neither the TCT nor reptilase time detects factor XIII deficiency. *HMWK*, High-molecular-weight kininogen (Fitzgerald factor); *NCS*, negatively charged surface; *Pre-K*, prekallikrein (Fletcher factor); *PL*, phospholipid; *TF*, tissue factor; *Thr*, thrombin (activated factor II, or IIa; serine protease); *Va*, *VIIIa*, activated factors V and VIII (serine protease cofactors); *VIIa*, *IXa*, *Xa*, *XIa*, activated factors VII, IX, X, XI (serine proteases); *XIIa*, activated factor XII (serine protease, but not part of in vivo coagulation); *XIIIa*, activated factor XIII (transglutaminase).

A TCT modification, the plasma-diluted TCT, provides a quantitative measure of dabigatran when used with calibrators of specific drug concentrations.⁷⁷

The fibrinogen assay described in a subsequent section is a simple modification of the TCT. In the fibrinogen assay, the concentration of reagent thrombin is 50 NIH units/mL, or about 10 times that used in the TCT, and the patient specimen is diluted 1:10. This dilution minimizes the effects of heparin or antithrombotic proteins. The reptilase time procedure described below is identical to the TCT procedure, except that the reptilase reagent is insensitive to the effects of heparin.

Reptilase Time

Reptilase Time Reagent and Principle

Reptilase is a thrombin-like enzyme isolated from the venom of *Bothrops atrox* that catalyzes the conversion of fibrinogen to fibrin (Pefakit Reptilase Time; Pentapharm, Inc., Basel, Switzerland). In contrast to thrombin, this enzyme cleaves only fibrinopeptide A from the fibrinogen molecule, whereas thrombin cleaves both fibrinopeptides A and B.⁷⁸ The specimen requirements, procedure, and quality assurance protocol for the reptilase time test are the same as those for the TCT. The reagent is reconstituted with distilled water and is stable for 1 month when stored at 1° C to 6° C. Reptilase time reagent is a poison that may be fatal if it directly enters the bloodstream.

Reptilase Time Clinical Utility

Reptilase is insensitive to heparin but is sensitive to dysfibrinogenemia, which profoundly prolongs the assay time. The reptilase time test is also useful for detecting hypofibrinogenemia or dysfibrinogenemia in patients receiving heparin therapy. The reptilase time is prolonged in the presence of FDPs and paraproteins.

Russell Viper Venom

Russell viper venom (RVV) from the *Daboia russelii* viper, which triggers coagulation at the level of factor X, was once used as an alternative to the prothrombin time. The assay was named the Stypven time, but is now obsolete. Russell viper venom is used in a dilute form to detect and confirm lupus anticoagulant, an assay called the dilute Russell viper venom time described in Chapter 39.

COAGULATION FACTOR ASSAYS

Fibrinogen Assay

Fibrinogen Assay Principle

The clot-based method of Clauss, a modification of the TCT, is the recommended procedure for estimating the functional fibrinogen level.^{79,80} The operator adds reagent bovine thrombin to dilute PPP, catalyzing the conversion of fibrinogen to fibrin polymer. In the fibrinogen assay, the thrombin reagent concentration is 50 NIH units/mL. The PPP to be tested is diluted 1:10 with Owren buffer. There is an inverse relationship between the interval to clot formation and the concentration of functional fibrinogen. Because the thrombin reagent is

concentrated and the PPP is diluted, the relationship is linear when the fibrinogen concentration is 100 to 400 mg/dL. Diluting the PPP also minimizes the antithrombotic effects of heparin, FDPs, and paraproteins; heparin levels less than 0.6 units/mL and FDP levels less than 100 µg/dL do not affect the results of the fibrinogen assay provided the fibrinogen concentration is 150 mg/dL or greater.

The interval to clot formation is compared with the results for fibrinogen calibrators. A calibration curve is prepared in each laboratory and updated regularly.

Fibrinogen Assay Procedure

Fibrinogen Assay Thrombin Reagent. Most laboratory managers prefer commercially manufactured diagnostic lyophilized bovine thrombin reagent for fibrinogen assays. Pharmaceutical topical thrombin also may be used. The reagent is reconstituted according to manufacturer instructions and used immediately or aliquotted and frozen. If thrombin is to be frozen, it should be prepared in a stock solution of 1000 NIH units/mL and frozen at -70° C until it is ready for use. When thawed, the thrombin is diluted 1:2 with buffer, is stable for only a few hours, and cannot be refrozen.

Fibrinogen Assay Calibration Curve. The laboratory practitioner prepares a calibration curve every 6 months at a minimum and with each change of reagent lot numbers, with a shift in QC, and after major maintenance. The curve is prepared by reconstituting commercially available lyophilized fibrinogen calibration plasma. Using Owren buffer, five dilutions of the calibration plasma are prepared: 1:5, 1:10, 1:15, 1:20, and 1:40. An aliquot of each dilution, usually 200 µL, is transferred to each of three reaction tubes or cups, warmed to 37° C, and tested by adding 100 µL of working thrombin reagent at 50 NIH units/mL. Time from addition of thrombin to clot formation is recorded, results of duplicate tests are averaged, and the values in seconds are graphed against fibrinogen concentration (Figure 42-11). Because patient PPPs are diluted 1:10 before testing, the 1:10 calibration plasma dilution is assigned the same fibrinogen concentration value as that of the undiluted reconstituted calibration plasma.

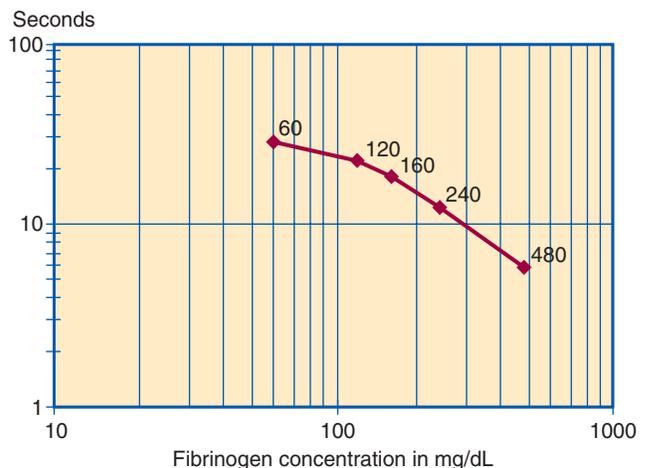


Figure 42-11 Fibrinogen calibrator curve plotted on log-log axes.

Fibrinogen Assay Test Protocol. The laboratory practitioner prepares a 1:10 dilution of each patient PPP and control with Owren buffer. Then 200 μL of each of the diluted PPPs is warmed to 37° C in each of two reaction tubes or cups for 3 minutes. After incubation, 100 μL of thrombin reagent is added, a timer is started, and the mixture is observed until a clot forms. The timer is stopped, values for duplicate runs are averaged, and the interval in seconds is compared with the graph. Results are reported in mg/dL of fibrinogen.

If the clotting time of the patient PPP dilution is short, indicating a fibrinogen level greater than 480 mg/dL, a 1:20 dilution is prepared and tested. The resulting fibrinogen concentration from the graph must be multiplied by 2 to compensate for the dilution. If the clotting time of the original 1:10 patient PPP dilution is prolonged, indicating less than 200 mg/dL of fibrinogen, a 1:5 dilution is prepared. The operator divides the resulting concentration reading from the graph by 2 to compensate for the greater concentration of the specimen.

Fibrinogen Assay Quality Control

All results for duplicate tests must agree within a coefficient of variation of less than 7%. The medical laboratory practitioner tests a normal control sample and an abnormal control sample with each batch of specimens for which fibrinogen levels are measured and records the results. The normal control results should be within the laboratory's reference interval. The abnormal control results should be less than 100 mg/dL. If either control result falls outside the control limits, the reagents, control, and equipment are checked; the problem is corrected; and the control is retested. The actions taken to correct out-of-limit tests are recorded. Control results are analyzed at regular intervals (weekly is typical) to determine the longitudinal validity of the procedure.

Specimen errors that affect the PTT likewise affect the fibrinogen assay and all factor assays (Table 42-7).

Fibrinogen Assay Results and Clinical Utility

One institution's reference interval for fibrinogen concentration is 220 to 498 mg/dL, although each local institution prepares its own interval. Hypofibrinogenemia, a fibrinogen level of less than 220 mg/dL, is associated with DIC and severe liver disease. Moderately severe liver disease, pregnancy, and a chronic inflammatory condition may cause an elevated fibrinogen level, greater than 498 mg/dL. Congenital afibrinogenemia leads to prolonged clotting times and is associated with a variable hemorrhagic disorder. Dysfibrinogenemia may give the same results as hypofibrinogenemia by this test method, because some abnormal fibrinogen species are hydrolyzed more slowly by thrombin than is normal fibrinogen. Some forms of dysfibrinogenemia may be associated with thrombosis.⁸¹

Fibrinogen values measured using immunologic assays and turbidimetric methods (Ellis-Stransky technique; PT-Fibrinogen HS Plus, Instrumentation Laboratory, Bedford, MA) are normal in dysfibrinogenemia. The fibrinogen concentration is estimated from reaction mixture turbidity and reported with each PT.

Fibrinogen Assay Limitations

Although antithrombotic effects are minimized by the dilution of PPP specimens, heparin levels greater than 0.6 units/mL and FDP levels greater than 100 $\mu\text{g}/\text{mL}$ prolong the results and give falsely lowered fibrinogen results. The operator ensures that the thrombin reagent is pure and has not degenerated. Exposure to sunlight or oxidation results in rapid breakdown. The working dilution lasts only 1 hour at 1° C to 6° C and should remain cold until just before testing.

Single-Factor Assays Using the Partial Thromboplastin Time Test

Principle of Single-Factor Assays Based on Partial Thromboplastin Time

If the PTT is prolonged and the PT and TCT are normal, and there is no ready explanation for the prolonged PTT such as heparin therapy, LA, or a factor-specific inhibitor, the medical laboratory practitioner may suspect a congenital single-factor deficiency. Three factor deficiencies that give this reaction pattern and cause hemorrhage are factor VIII deficiency (hemophilia A), factor IX deficiency (hemophilia B), and factor XI deficiency, which causes a mild intermittent bleeding disorder called *Rosenthal syndrome* found primarily in Ashkenazi Jews.^{82,83} These deficiencies are most often detected in childhood. The next step in diagnosis of a congenital single-factor deficiency is the performance of a one-stage single-factor assay based on the PTT system.

Although necessary for diagnosis, PTT-based single-factor assays are most often performed on specimens from patients with previously identified single-factor deficiencies. Their purpose is to monitor supportive therapy during bleeding episodes or invasive procedures. Because hemophilia A is the most common single-factor deficiency disorder, this discussion is confined to the factor VIII assay; however, the protocol may be generalized to the assays for factors IX and XI.

The medical laboratory practitioner uses the PTT system to estimate the concentration of functional factor VIII by incorporating commercially prepared factor VIII-depleted PPP in the test system (*Cryocheck* Factor VIII Deficient Plasma; Precision BioLogic Inc, Dartmouth, Nova Scotia). Distributors collect plasma from normal donors and employ *immunodepletion*, relying on a monoclonal anti-factor VIII antibody bound to a separatory column, to prepare factor VIII-depleted plasma.⁸⁴

In the PTT-based factor assay system, factor VIII-depleted PPP provides normal activity of all procoagulants except factor VIII. Tested alone, factor VIII-depleted PPP has a prolonged PTT, but when normal PPP is added, the PTT reverts to normal. In contrast, a prolonged result for a mixture of patient PPP and factor VIII-depleted PPP implies that the patient PPP is factor VIII deficient. The clotting time interval for the mixture of patient PPP and factor VIII-depleted PPP may be compared with a previously prepared reference curve to estimate the level of factor VIII activity in the patient PPP. The quantitative factor assay is typically performed on three or four dilutions of patient PPP—for instance, 1:10, 1:20, 1:40, and 1:80—and the results compared with mathematical manipulation. Multiple dilutions contribute to the accuracy of the results.

Factor VIII Assay Reference Curve

To prepare a reference curve for the factor VIII assay, the laboratory practitioner obtains a reference plasma such as CAP FVIIIc RM (College of American Pathologists, Northfield, IL) and prepares a series of dilutions with buffered saline.⁸⁵ Although laboratory protocols vary, most laboratory practitioners prepare a series of five dilutions, from 1:5 to 1:500. Each dilution is mixed with reagent factor VIII-depleted plasma and tested in duplicate using the PTT system. The duplicate results are averaged and plotted on log-log or log-linear graph paper (Figure 42-12). The 1:10 dilution is assigned the factor VIII assay activity value found on the package insert. When patient PPP is tested, the time interval obtained is entered on the vertical coordinate and converted to a percentage.⁸⁶

Factor VIII Assay Procedure

The medical laboratory practitioner (or the automated coagulometer) prepares 1:10, 1:20, 1:40, and 1:80 dilutions of each patient PPP and control specimen and then mixes each dilution with equal volumes of factor VIII-depleted plasma and PTT reagent. In most cases, 100 μ L of PTT reagent is mixed with 100 μ L each of patient PPP dilution and factor VIII-depleted plasma mixture. All dilutions of each specimen or control are tested in duplicate. After incubation at 37° C for the manufacturer-specified time, typically 3 minutes, 100 μ L of 0.025 M calcium chloride is added, and a timer is started. The interval is recorded in seconds, duplicates are averaged, the mean result is compared with the reference curve, and the percentage of factor VIII activity is reported. Factor activity results for the 1:20, 1:40, and 1:80 dilutions are multiplied by 2, 4, and 8, respectively, to compensate for the dilutions and should match the results of the 1:10 dilutions within 10%. If the results of the dilutions do not match within 10%, they are considered to be *nonparallel*. An LA may be present, and the assay cannot provide a reliable estimate of factor VIII activity.

Tests for factors IX and XI are performed using the same approach, except that the appropriate factor-depleted plasma is substituted for factor VIII-depleted plasma. Tests for the contact factors XII, prekallikrein, and high-molecular-weight kininogen are seldom requested because deficiencies are not associated with bleeding disorders. Acquired and congenital contact factor deficiencies are relatively common, however, and

cause PTT prolongation. Factor XII, prekallikrein, and high-molecular-weight kininogen assays are available from hemostasis reference laboratories, and their use may be necessary to account for an unexplained prolonged PTT.

Expected Results and Clinical Utility of Single-Factor Assays

The reference interval for factor VIII activity is 50% to 186%. Spontaneous symptoms of hemophilia are evident at activity levels of 10% or less. The test is used most often to estimate the plasma level of factor VIII activity during therapy (Chapter 38). Chronically elevated factor VIII predicts an elevated risk of venous thrombotic disease (Chapter 39).

Single-Factor Assay Quality Control

All duplicate results must agree within 10%. The medical laboratory practitioner tests a normal and a deficient control specimen with each assay and records the results. The normal control results should fall within the reference interval. The deficient control results should be in the range of 10% factor VIII activity or below. If either control result falls outside the control limits, the reagents, control, and equipment are checked; the problem is corrected; and the control is retested. The practitioner records all actions taken to correct out-of-limit tests. Control results are analyzed at regular intervals (weekly is typical) to determine the longitudinal validity of the procedure.

Limitations of Single-Factor Assays

Interlaboratory coefficients of variation for the factor VIII assay reach 80%, which implies undesirable variation in the interpretation of therapeutic monitoring results from unrelated institutions. To reduce inherent variation, the medical laboratory practitioner uses assayed commercial plasma to prepare the reference curve and selects reference dilutions that correspond to only the linear portion of the curve. The laboratory must assay three or more dilutions of patient PPP to check for inhibitors. The practitioner also selects a matching reagent-instrument system with a demonstrated coefficient of variation of less than 5% and uses factor-depleted substrates with no trace of the depleted factor.⁸⁷ As with the PTT test, good specimen management is essential. Clotted, hemolyzed, icteric, or lipemic specimens are rejected because they give unreliable results. Reagents must be reconstituted with the correct diluents and volumes following manufacturer instructions. Reagents must be stored and shipped in accordance with manufacturer instructions and never used after the expiration date.

Bethesda Titer for Anti-Factor VIII Inhibitor

The Bethesda titer is used to confirm the presence of and quantify an anti-factor VIII inhibitor, which is typically an IgG4-class immunoglobulin.⁸⁸ In this method, 200 μ L of patient PPP is incubated with 200 μ L of reagent normal plasma for 2 hours at 37° C. A control specimen consisting of 200 μ L of imidazole buffer at pH 7.4 mixed with 200 μ L of reagent normal plasma is incubated simultaneously. During the incubation period,

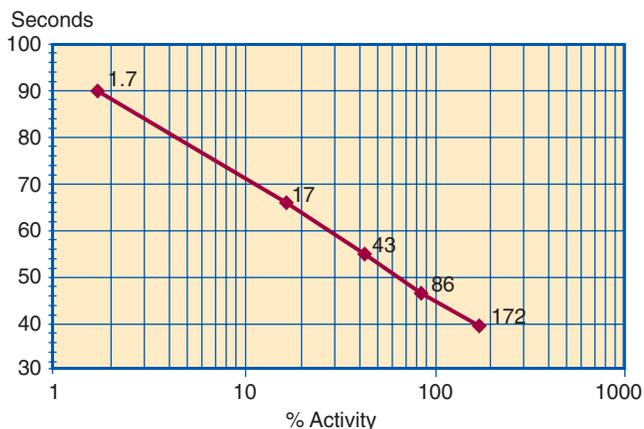


Figure 42-12 Factor VIII assay calibrator curve plotted on linear-log axes.

anti-factor VIII from the patient PPP neutralizes a percentage of the reagent normal plasma factor VIII activity. The degree of factor VIII activity neutralized is proportional to the level of inhibitor activity. After incubation, residual factor VIII activity in the patient PPP–reagent normal plasma mixture is measured using the specific factor activity assay as described in the section on factor assays using the PTT system.

The titer of inhibitor is expressed as a percentage of the control. If the patient PPP–reagent normal plasma mixture retains 75% of the residual factor VIII activity of the control, no factor VIII inhibitor is present. If the residual factor VIII level is 25% that of the control, the patient PPP factor VIII inhibitor level is titered using several dilutions of the patient specimen in reagent normal PPP. One Bethesda unit of activity is the amount of antibody that leaves 50% residual factor VIII activity in the mixture.

Single-Factor Assays Using the Prothrombin Time Test

If the PTT and the PT are both prolonged, the TCT is normal, and there is no ready explanation for the prolonged test results, such as liver disease, vitamin K deficiency, DIC, or Coumadin therapy, the medical laboratory practitioner may suspect a congenital single-factor deficiency of the common pathway (Chapter 37). Three relatively rare factor deficiencies that give this reaction pattern and cause hemorrhage are prothrombin deficiency, factor V deficiency, and factor X deficiency. If the PT is prolonged and all other test results are normal, factor VII deficiency is suspected. The next step is the performance of a one-stage single-factor assay based on the PT test system, which is a relatively rare event. The principles and procedure described in the section on single-factor assay using the PTT system may be applied except that PT reagent replaces the PTT reagent in the test system, and the PT protocol is followed. Factor II (prothrombin)-depleted, factor V-depleted, factor VII-depleted, and factor X-depleted plasmas are available (Table 42-8).

Factor XIII Assay

Coagulation factor XIII is a transglutaminase that catalyzes covalent cross-links between the α and γ chains of fibrin polymer.⁸⁹ Cross-linking strengthens the fibrin clot and renders it

resistant to proteases. This is the final event in coagulation, and it is essential for normal hemostasis and normal wound healing. Factor XIII from plasma, platelets, and tissue function identically. Neither the PT nor the PTT is prolonged by factor XIII deficiency.

Inherited factor XIII deficiency, an autosomal recessive disorder, affects both sexes in all races. The first report of the deficiency appeared in 1960, and the frequency is estimated at 1 in 2 million. Factor XIII levels also may be low in chronic DIC secondary to Crohn disease, leukemias, ulcerative colitis, sepsis, inflammatory bowel disease, surgery, and Henoch-Schönlein purpura. In these cases, the factor XIII level decreases to 50% of normal, not low enough to create symptoms, although occasionally acquired factor XIII deficiencies produce low enough levels to cause mild bleeding. Acquired factor XIII inhibitors have been described in patients treated with isoniazid, penicillin, valproate, and phenytoin.⁹⁰ These drugs may cause complete absence of factor XIII.

Factor XIII activity levels lower than 5% result in hemorrhage. In congenital factor XIII deficiency, bleeding is evident in infants, with seepage at the umbilical stump.⁹¹ In adults, bleeding is slow but progressive, accompanied by poor wound healing and slowly resolving hematomas. Recurrent spontaneous abortion and posttraumatic hemorrhage are common. Acquired factor XIII inhibitors cause severe bleeding that does not respond to therapy.

When a patient comes for treatment of bleeding and poor wound healing and the PTT, PT, platelet count, and fibrinogen level are normal, the laboratory practitioner may recommend a factor XIII assay such as the Technochrom Factor XIII (DiaPharma Group, Inc., West Chester, OH).⁹² In this representative assay, quantitation of factor XIII activity is based on the measurement of ammonia released during an *in vitro* transglutaminase reaction. Plasma factor XIII is first activated by reagent thrombin. The resultant factor XIIIa then cross-links the fibrin amine substrate glycine ethyl ester to the glutamine residue of a peptide substrate, releasing ammonia. The concentration of ammonia is monitored in a glutamate dehydrogenase-catalyzed reaction that depends on NADPH, the reduced form of nicotinamide adenine dinucleotide phosphate. NADPH consumption is measured by the decrease of absorbance at 340 nm. The absorbance is inversely proportional to factor XIII activity. Several manufacturers market immunoassays for factor XIII, which provide factor XIII concentration but do not identify functional factor XIII abnormalities.

TABLE 42-8 Factor Assays Using the TCT, PT, and PTT Test Systems

Factor	System
Fibrinogen (I)	Clauss method: modified thrombin clotting time
Prothrombin (II)	Prothrombin time
Factor V	Prothrombin time
Factor VII	Prothrombin time
Factor VIII	Partial thromboplastin time
Factor IX	Partial thromboplastin time
Factor X	Prothrombin time
Factor XI	Partial thromboplastin time
Factor XIII	Chromogenic assay

PT, prothrombin time; PTT, partial thromboplastin time; TCT, thrombin clotting time.

TESTS OF FIBRINOLYSIS

Quantitative D-Dimer Immunoassay Physiology of Fibrin Degradation Products and D-Dimers

During coagulation, fibrin polymers become cross-linked by factor XIIIa and simultaneously bind plasma plasminogen and tissue plasminogen activator (TPA) (Chapter 37). Over several hours, bound TPA activates nearby plasminogen to form plasmin. The bound plasmin cleaves fibrin and yields the FDPs D, E, X, and Y and D-dimer. The FDPs represent

original fibrinogen domains, and D-dimers are covalently linked D domains reflecting the cross-linking effects of factor XIIIa. Assays for FDPs, including D-dimer, are convenient for detecting active fibrinolysis, which indirectly implies the occurrence of thrombosis. Normally, FDPs, including D-dimer, circulate at concentrations of less than 2 ng/mL. Fibrinolysis yields FDPs and D-dimer at concentrations greater than 200 ng/mL. Increased FDP and D-dimer concentrations are characteristic of acute and chronic DIC, systemic fibrinolysis, deep vein thrombosis, and pulmonary embolism.⁹³ FDPs, including D-dimer, also are detected in plasma after thrombolytic therapy.⁹⁴

Principle of the Quantitative D-Dimer Assay

Plasma D-dimer immunoassays abound, and several diagnostics distributors offer automated quantitative immunoassays for plasma D-dimers that generate results within 30 minutes.⁹⁵ Microlatex particles in buffered saline are coated with monoclonal anti-D-dimer antibodies. The coated particles are agglutinated by patient plasma D-dimer; the resultant turbidity is measured using turbidometric or nephelometric technology. Sensitivity varies, depending on the avidity of the monoclonal anti-D-dimer and the detection method; however, most methods detect concentrations as low as 10 ng/mL.

Clinical Value of the Quantitative D-Dimer Assay

The quantitative D-dimer assay is essential for ruling out venous thromboembolic disease in patients with low pretest probability and is required for detecting and monitoring DIC (Chapter 39).⁹⁶ The D-dimer assay helps rule out acute myocardial infarction and ischemic stroke and may be used to monitor the efficacy of Coumadin therapy.⁹⁷ The various quantitative D-dimer assays have negative predictive values of 90% to 95% and may be used to rule out deep vein thrombosis and pulmonary thrombotic emboli in patients at low risk without resorting to compression ultrasonography, tomography, or venous imaging.^{98,99} Because of the high sensitivity but low specificity (60% to 70%) of the quantitative D-dimer assay, laboratory practitioners do not use this assay to positively diagnose venous thromboembolic disease but only to rule it out. Because any chronic or acute inflammation is accompanied by elevated D-dimer concentrations, the assay cannot be used to “rule in” thromboembolic disease. The upper limit of the reference interval for the quantitative D-dimer assay varies with the methodology, ranging from 250 ng/mL to 500 ng/mL. In DIC, D-dimer levels may reach 10,000 to 20,000 ng/mL.

Qualitative D-Dimer Assay

The automated quantitative D-dimer assay has largely replaced manual D-dimer or FDP assays. The SimpliRED D-dimer assay (BBInternational, Inc., Dundee, United Kingdom) is a manual method that uses visible latex particles coated with monoclonal antibody. The SimpliRED D-dimer assay is suited to low-volume or near-patient (point-of-care) applications. The manufacturer reports the clinical sensitivity for pulmonary embolus to be 94% and the specificity to be 67%.¹⁰⁰

Fibrin Degradation Product Immunoassay

Although the FDP assay has largely been replaced by the automated quantitative D-dimer assay or the manual semiquantitative D-dimer assay, FDPs may be detected using a semiquantitative visible agglutination immunoassay.¹⁰¹ One such method is the 1972 Thrombo-Wellcotest (Remel, Inc., Lenexa, KS).¹⁰² Polystyrene latex particles in buffered saline are coated with polyclonal antibodies specific for D and E fragments calibrated to detect FDPs at a concentration of 2 µg/mL or greater. The assay usually is performed on serum collected in special tubes that promote clotting and prevent *in vitro* fibrinolysis, although plasma-based assays are also available.

Plasminogen Chromogenic Substrate Assay

Excessive fibrinolytic activity occurs in a variety of conditions. Inflammation and trauma may be reflected in a radical increase in circulating plasmin that has the potential to cause hemorrhage. Bone trauma, fractures, and surgical dissection of bone, as in cardiac surgery, may raise fibrinolysis activity.¹⁰³ Fibrinolysis deficiencies occur when TPA or plasminogen levels become depleted or when excess secretion of PAI-1 depresses TPA activity. Plasminogen, the precursor of the trypsin-like proteolytic enzyme plasmin, is produced in the liver and circulates as a single-chain glycoprotein (Chapter 37). When bound to fibrin, plasminogen is converted to plasmin by the action of nearby TPA. Bound plasmin degrades fibrin, whereas a circulating inhibitor, α_2 -antiplasmin, rapidly inactivates free plasmin.

Congenital plasminogen deficiencies are associated with thrombosis in some families.¹⁰⁴ Acquired plasminogen deficiencies are seen in DIC and acute promyelocytic leukemia.¹⁰⁵ Thrombolytic therapy is ineffective when plasminogen activity is low. Plasminogen is readily measured in PPP using a chromogenic substrate assay, available from several manufacturers.

Principle of the Plasminogen Chromogenic Substrate Assay

Chromogenic substrates employ synthetic oligopeptides whose amino acid sequences are designed to be specific for their chosen enzymes. Plasmin hydrolyzes a bond in the oligopeptide sequence valine-leucine-lysine (Val-Leu-Lys). A fluorophore or a chromophore such as para-nitroaniline (pNA) is covalently bound to the carboxyl terminus of the oligopeptide and may be released on digestion. S-2251, composed of H-D-Val-Leu-Lys-pNA, is a chromogenic substrate for plasmin. On plasmin digestion, the pNA is released and transforms from a colorless liquid to yellow (Figure 42-13).

Streptokinase is an exogenous plasminogen activator derived from cultures of β -hemolytic streptococci. Streptokinase is added to patient PPP, where it binds and activates plasminogen. The resulting streptokinase-plasmin complex reacts with a chromogenic substrate such as S-2251 to release a color whose intensity is proportional to the plasminogen concentration. Several analogous chromogenic and fluorogenic substrates are suitable for plasminogen measurement. Control plasma is tested with the patient plasma, and the results are recorded.

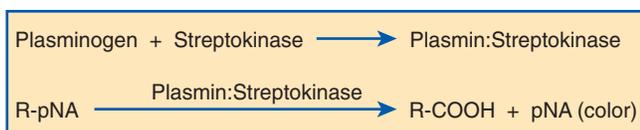


Figure 42-13 Assay of plasma plasminogen using the chromogenic substrate method. Reagent streptokinase activates plasminogen to form plasmin. *R-pNA* designates a chromogenic substrate, where *R* indicates one of several choices of peptide sequence and *pNA* (para-nitroaniline) is the chromophore. In the case of plasminogen, the *R* represents the peptide sequence valine-leucine-lysine (Val-Leu-Lys). Plasmin recognizes the Val-Leu-Lys amide sequence as its enzymatic cleavage site, releasing the pNA, which generates a yellow color.

Results and Clinical Utility of the Plasminogen Chromogenic Substrate Assay

The plasminogen reference interval is 5 to 13.5 mg/dL. Plasminogen levels are decreased in thrombolytic therapy, DIC, hepatitis, and cancer. Hereditary deficiencies have also been recorded.¹⁰¹ Decreased plasminogen is associated with thrombosis. Plasminogen rises in inflammation and during pregnancy, and high levels may be associated with hemorrhage. Plasminogen levels may also be elevated in systemic fibrinolysis. Plasminogen assays are seldom offered in acute care facilities but are readily available at specialty reference laboratories.

Tissue Plasminogen Activator Assay

Physiology of Tissue Plasminogen Activator

The two physiologic human plasminogen activators are TPA and urokinase.^{106,107} TPA is synthesized in vascular endothelial cells and released into the circulation, where its half-life is approximately 3 minutes and its plasma concentration averages 5 ng/mL. Urokinase is produced in the kidney and vascular endothelial cells and has a half-life of approximately 7 minutes and a concentration of 2 to 4 ng/mL. Both activators are serine proteases that form ternary complexes with bound plasminogen at the surface of fibrin, activating the plasminogen and initiating thrombus degradation. The endothelial secretion *plasminogen activator inhibitor-1* (PAI-1) covalently inactivates both.

Clinical Significance of Tissue Plasminogen Activator

The reference interval upper limit for TPA activity is 1.1 units/mL, and the upper limit for TPA antigen is 14 ng/mL. TPA is the primary mediator of fibrinolysis and is the model for synthetic TPA (Activase; Genentech, Inc., South San Francisco, CA). Decreased TPA levels may indicate increased risk of myocardial infarction, stroke, or deep vein thrombosis, although more data are needed to verify a relationship.¹⁰⁸ Impaired fibrinolysis in the form of TPA deficiency or PAI-1 excess also is associated with deep vein thrombosis and myocardial infarction.¹⁰⁹

Specimen Collection for the Tissue Plasminogen Activator Assay

TPA activity exhibits diurnal variation and rises upon exercise. Further, TPA is unstable in vitro because it rapidly binds PAI-1

after collection. For specimen collection, patients should be at rest, tourniquet application should be minimal, the phlebotomist should record the collection time, and immediate acidification of the specimen in acetate buffer is necessary.¹¹⁰ Acidification may be accomplished using the Stabilyte acidified citrate tube (Diagnostica Stago, Inc., Parsippany, NJ). Supernatant PPP may be frozen at -70°C until the assay is performed.

Principle of the Tissue Plasminogen Activator Assay

Plasma concentration of TPA antigen may be estimated by enzyme immunoassay. To measure TPA activity, a specified concentration of reagent plasminogen is added to the patient plasma (Chromolyse TPA Activity; Diagnostica Stago, Inc, Parsippany, NJ). Plasma TPA activates the plasminogen, and the resultant plasmin activity is measured using a chromogenic substrate. The resulting color intensity is proportional to TPA activity (Figure 42-14). The system may incorporate soluble fibrin to increase TPA activity.

Plasminogen Activator Inhibitor-1 Assay

PAI-1 is produced by vascular endothelial cells and hepatocytes and circulates in plasma bound to vitronectin at an average concentration of 10 $\mu\text{g/L}$ with diurnal variations.¹¹¹ An inactive form of PAI-1 circulates in high concentrations in platelets.¹¹² PAI-1 inactivates free TPA by covalent binding. Elevated PAI-1 is associated with venous thrombosis and may be a cardiovascular risk factor (Chapter 39). A few cases of PAI-1 deficiency have been reported; however, hemorrhage apparently occurs only in the complete absence of PAI-1.

Blood is collected from patients at rest into an acidified citrate tube (Stabilyte) and centrifuged immediately to make PPP; this avoids in vitro release of platelet PAI-1. Several immunometric and chromogenic substrate methods are available for estimation of PAI-1 antigen and PAI-1 activity, respectively. One enzyme immunoassay for functional PAI-1 uses urokinase to bind PAI-1. The urokinase-PAI-1 complex is immobilized with solid-phase monoclonal anti-PAI-1 and is measured using monoclonal anti-urokinase immunoglobulin as the detecting antibody.^{113,114}

Most chromogenic substrate kits for PAI-1 use an indirect measurement approach (Spectrolyse PAI-1; Diagnostica Stago, Inc, Parsippany, NJ). Patient PPP is mixed with a measured amount of reagent TPA. Residual TPA is assayed in the

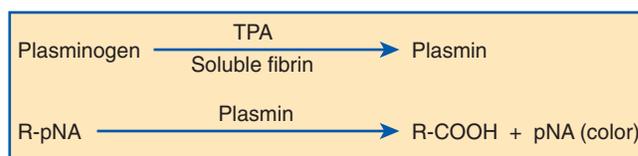


Figure 42-14 To assay tissue plasminogen activator (TPA), plasma that contains TPA is added to plasminogen to produce plasmin. Plasmin activity is measured using the same chromogenic substrate system as in the plasminogen assay illustrated in Figure 42-13. The intensity of color is proportional to TPA activity. *pNA*, Para-nitroaniline; *R*, variable peptide sequence.

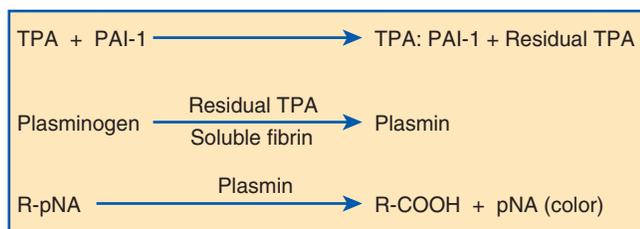


Figure 42-15 To assay plasminogen activator inhibitor 1 (PAI-1) activity, plasma containing PAI-1 is added to reagent tissue plasminogen activator (TPA) of known concentration. The residual TPA is assayed, as shown in Figure 42-14. The intensity of color is inversely proportional to PAI-1 level. *pNA*, Para-nitroaniline; *R*, variable peptide sequence.

plasminogen system as shown in Figure 42-15. The resulting color intensity is inversely proportional to plasma PAI-1 activity.

Confirmation of total PAI-1 deficiency may be accomplished using the *serum* PAI-1 assay. In serum, platelet PAI-1 is expressed in excess. In true PAI-1 absence, no PAI-1 is detectable in serum.

GLOBAL COAGULATION ASSAYS

The TEG Thromboelastograph Hemostasis Analyzer (Haemoscope Corporation, Niles, IL, a division of Haemonetics Corporation) and the ROTEM (Tem, Inc, Durham, NC) are global whole-blood analyzers that measure clotting time and dynamics, clot strength, antithrombotic effects, platelet effects on clot dynamics and strength, and fibrinolysis.¹¹⁵ Both are used as a manual coagulometers, mainly in liver and cardiac surgeries and are described in detail in Chapter 44.

SUMMARY

- Proper hemostasis specimen collection, transport, storage, and centrifugation ensure a valid test result.
- Specimens that are short draws, clotted, or hemolyzed are rejected.
- Platelet aggregometry helps determine the cause of nonthrombocytopenic mucocutaneous bleeding.
- VWD is diagnosed and monitored through the judicious selection and performance of platelet-based laboratory tests.
- The plasma markers PF4 and β -thromboglobulin are research applications used to assess platelet activation.
- Clot-based coagulation screening tests include the ACT, PT, PTT, and TCT.
- Mixing studies are used to detect factor deficiencies, LAs, and specific factor inhibitors.
- Coagulation factor assays are used to detect and measure coagulation factor deficiencies.
- Bethesda titers are used to detect and measure coagulation factor inhibitors.
- Tests of fibrinolysis include assays for FDPs, D-dimer, plasminogen, TPA, and PAI-1.
- Thromboelastography and thromboelastometry are widely used global hemostasis assay methods.

Now that you have completed this chapter, go back and read again the case study at the beginning and respond to the questions presented.

REVIEW QUESTIONS

Answers can be found in the Appendix.

1. What happens if a coagulation specimen collection tube is underfilled?
 - a. The specimen clots and is useless
 - b. The specimen is hemolyzed and is useless
 - c. Clot-based test results are falsely prolonged
 - d. Chromogenic test results are falsely decreased
2. If you collect blood into a series of tubes, when in the sequence should the hemostasis (blue stopper) tube be filled?
 - a. After a lavender-topped or green-topped tube
 - b. First, or after a nonadditive tube
 - c. After a serum separator tube
 - d. Last
3. What is the effect of hemolysis on a hemostasis specimen?
 - a. In vitro platelet and coagulation activation occur
 - b. The specimen is icteric or lipemic
 - c. Hemolysis has no effect
 - d. The specimen is clotted
4. Most coagulation testing must be performed on PPP, which is plasma with a platelet count less than:
 - a. 1000/ μL
 - b. 10,000/ μL
 - c. 100,000/ μL
 - d. 1,000,000/ μL

5. You wish to obtain a 5-mL specimen of whole-blood/anticoagulant mixture. The patient's hematocrit is 65%. What volume of anticoagulant should you use?
 - a. 0.32 mL
 - b. 0.5 mL
 - c. 0.64 mL
 - d. 0.68 mL
6. You perform whole-blood lumiaggregometry on a specimen from a patient who complains of easy bruising. Aggregation and secretion are diminished when the agonists, thrombin, ADP, arachidonic acid, and collagen are used. What is the most likely platelet abnormality?
 - a. Storage pool disorder
 - b. Aspirin-like syndrome
 - c. ADP receptor anomaly
 - d. Glanzmann thrombasthenia
7. What is the reference assay for HIT?
 - a. Enzyme immunoassay
 - b. Serotonin release assay
 - c. Platelet lumiaggregometry
 - d. Washed platelet aggregation
8. What agonist is used in platelet aggregometry to detect VWD?
 - a. Arachidonic acid
 - b. Ristocetin
 - c. Collagen
 - d. ADP
9. Deficiency of which single factor is likely when the PT result is prolonged and the PTT result is normal?
 - a. Factor V
 - b. Factor VII
 - c. Factor VIII
 - d. Prothrombin
10. A prolonged PT, a low factor VII level, but a normal factor V level are characteristic of an acquired coagulopathy associated with which of the following?
 - a. Hemophilia
 - b. Liver disease
 - c. Thrombocytopenia
 - d. Vitamin K deficiency
11. The patient has deep vein thrombosis. The PTT is prolonged and is not corrected in an immediate mix of patient plasma with an equal part of normal plasma. What is the presumed condition?
 - a. Factor VIII inhibitor
 - b. Lupus anticoagulant
 - c. Factor VIII deficiency
 - d. Factor V Leiden mutation
12. What condition causes the most pronounced elevation in the result of the quantitative D-dimer assay?
 - a. Deep vein thrombosis
 - b. Fibrinogen deficiency
 - c. Paraproteinemia
 - d. DIC
13. What is the name given to the type of assay that uses a synthetic polypeptide substrate that releases a chromophore on digestion by its serine protease?
 - a. Clot-based assay
 - b. Molecular diagnostic assay
 - c. Fluorescence immunoassay
 - d. Chromogenic substrate assay
14. What component of the fibrinolytic process binds and neutralizes free plasmin?
 - a. PAI-1
 - b. TPA
 - c. α_2 -Antiplasmin
 - d. Urokinase

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43

Antithrombotic Therapies and Their Laboratory Assessment

George A. Fritsma

OUTLINE

Coumadin Therapy and the Prothrombin Time

Coumadin Is a Vitamin K Antagonist

Coumadin Prophylaxis and Therapy

Monitoring Coumadin Therapy Using the Prothrombin Time Assay

Monitoring Coumadin Therapy Using the Chromogenic Factor X Assay

Effect of Diet and Drugs on Coumadin Therapy

Effect of Polymorphisms on Coumadin Therapy

Effect of Direct Thrombin Inhibitors on the Prothrombin Time

Reversing Bleeding Caused by a Coumadin Overdose

Unfractionated Heparin Therapy and the Partial Thromboplastin Time

Heparin Is a Catalyst That Activates Antithrombin to Neutralize Serine Proteases

Unfractionated Heparin Therapy

Monitoring Unfractionated Heparin Therapy Using the Partial Thromboplastin Time

Determining the Partial Thromboplastin Time Therapeutic Range for Unfractionated Heparin Therapy

Clinical Utility of Monitoring Unfractionated Heparin Therapy Using the Partial Thromboplastin Time

OBJECTIVES

After completion of this chapter, the reader will be able to:

1. Describe the purpose of antithrombotic drug administration and distinguish between anticoagulants and antiplatelet therapy.
2. Describe the indications for, dosage of, and management of Coumadin therapy, including how to detect and manage Coumadin overdose.
3. Monitor Coumadin therapy using the prothrombin time and international normalized ratio, and compare these tests with the chromogenic factor X assay.
4. Perform and interpret prothrombin times with international normalized ratios using point-of-care instruments.
5. Describe the indications for, dosage of, and laboratory monitoring of unfractionated heparin therapy, including how to establish the unfractionated heparin partial thromboplastin time therapeutic range.
6. Perform and interpret the results of the partial thromboplastin time and activated clotting time assays for monitoring unfractionated heparin therapy, and compare these tests with the chromogenic anti-factor Xa heparin assay.
7. Describe the indications, dosage, and laboratory measurement of low-molecular-weight heparin therapy and fondaparinux therapy.
8. Perform and interpret the results of the chromogenic anti-factor Xa assay for measuring unfractionated heparin, low-molecular-weight heparin, fondaparinux, rivaroxaban, apixaban, and edoxaban therapy.
9. Measure direct thrombin inhibitor therapy, including dabigatran, using the partial thromboplastin time, ecarin clotting time, ecarin chromogenic assay, and plasma-diluted thrombin time.
10. Describe the indications for intravenous platelet glycoprotein IIb/IIIa inhibitors abciximab, eptifibatide, and tirofiban and describe how their effects are measured.
11. Describe the indications for oral platelet inhibitors aspirin, clopidogrel, prasugrel, and ticagrelor, and describe how their effects are measured.

CASE STUDY

After studying the material in this chapter, the reader should be able to respond to the following case study:

A 71-year-old woman with atrial fibrillation has been taking 5 mg of Coumadin (warfarin sodium) per day for 8 years. Her monthly prothrombin time and international normalized ratio (PT/INR) has been maintained consistently within the standard therapeutic range, 2 to 3. Last Monday, however, her INR was 6.5. For several days earlier she had noticed that her gums bled after she brushed her teeth.

1. What could cause this change in the PT/INR result?
2. What should be done about her PT/INR and symptoms?
3. What alternative test may be used to monitor Coumadin therapy?

OUTLINE—cont'd

Limitations of Monitoring Unfractionated Heparin Therapy Using the Partial Thromboplastin Time
Monitoring Unfractionated Heparin Therapy Using the Activated Clotting Time
Reversal of Unfractionated Heparin Overdose Using Protamine Sulfate

Low-Molecular-Weight Heparin Therapy and the Chromogenic Anti-Factor Xa Heparin Assay

Low-Molecular-Weight Heparins Produced from Unfractionated Heparin
Measuring Low-Molecular-Weight Heparin Therapy

Measuring Pentasaccharide Therapy Using the Chromogenic Anti-Factor Xa Heparin Assay

Measuring Oral Direct Factor Xa Inhibitors
Direct Thrombin Inhibitors

Argatroban
Bivalirudin, a Recombinant Analogue of Leech Saliva Hirudin
Dabigatran, an Oral Direct Thrombin Inhibitor
Measuring Direct Thrombin Inhibitor Therapy

Measuring Antiplatelet Therapy Using Platelet Activity Assays

Intravenous Glycoprotein IIb/IIIa Inhibitors Are Used During Cardiac Catheterization
Aspirin, Clopidogrel, Prasugrel, and Ticagrelor Reduce the Incidence of Arterial Thrombosis
Variable Aspirin and Clopidogrel Response and Laboratory Measuring of Antiplatelet Resistance

Future of Antithrombotic Therapy

Thrombosis, described in detail in the introduction to Chapter 39, is the pathological formation of blood clots in veins or arteries that obstruct flow and cause tissue ischemia and necrosis. Antithrombotic drugs have been employed to treat and prevent thrombosis since heparin was first developed in 1916 and then was FDA-cleared in 1936.¹ Antithrombotics include *anticoagulants*, which suppress coagulation and reduce thrombin formation, and *antiplatelet drugs*, which suppress platelet activation. Fibrinolytics are also employed to disperse or reduce existing clots clogging veins and arteries. **Table 43-1** provides a list of current antithrombotics with their indications.

Venous thromboembolic disease (VTE, venous thromboembolism; Chapter 39) includes superficial and *deep vein thrombosis* (DVT) and *pulmonary embolism* (PE). VTE is treated using intravenous standard unfractionated heparin (UFH), subcutaneous low-molecular-weight heparin (LMWH, enoxaparin, tinzaparin), subcutaneous synthetic pentasaccharide (fondaparinux), or the oral direct factor Xa inhibitor, rivaroxaban. VTE is also treated using the oral vitamin K antagonist Coumadin (warfarin sodium). These anticoagulants are also used to prevent VTE subsequent to total hip and total knee replacement surgery, orthopedic repair surgery, and in several medical conditions.

The *direct thrombin inhibitors* (DTIs) *argatroban* and *bivalirudin* are intravenous anticoagulants that are substituted for UFH in patients who have developed *heparin-induced thrombocytopenia with thrombosis* (HIT), a devastating arterial and venous thrombotic side effect of UFH therapy (Chapters 39 and 40). Dabigatran is an oral DTI cleared in 2010 to prevent ischemic stroke, a common side effect for patients who suffer nonvalvular atrial fibrillation. The direct anti-factor Xa anticoagulants rivaroxaban and apixaban are also available to prevent ischemic stroke in atrial fibrillation.

Arterial thrombosis includes *acute myocardial infarction* (AMI), ischemic *cerebrovascular accident* (CVA, stroke), *transient ischemic attack* (TIA), and *peripheral arterial occlusion* (PAO or peripheral artery disease, PAD) and is managed with UFH, LMWH, fondaparinux, Coumadin, the intravenous DTIs, and the antiplatelet drugs aspirin, clopidogrel, prasugrel, and ticagrelor. Aspirin is taken prophylactically by many healthy people at less than 100 mg/day and is particularly effective in reducing mortality when taken within minutes of the acute onset of stroke or cardiac symptoms.² The intravenous platelet *glycoprotein IIb/IIIa inhibitor* (GPI) drugs eptifibatide, abciximab, and tirofiban are used to prevent thrombosis during cardiac catheterization procedures.

Thrombolytic therapy may be used to resolve DVT, PE, PAO, AMI, and stroke, particularly when used 3 to 4 hours after the onset of symptoms. Thrombolytic therapy employs recombinant forms of *tissue plasminogen activator* (reteplase, alteplase, and tenecteplase). Thrombolytic therapy raises the risk of hemorrhage, particularly intracranial hemorrhage. Because thrombolytic therapy is not measured by laboratory tests (although fibrinogen levels can be checked), it is not further discussed in this chapter.

Many lives have been saved through the judicious use of antithrombotic therapy, and countless more healthy individuals have been spared thrombotic disease through long-term antithrombotic prophylaxis in moderate-risk circumstances and conditions. However, antithrombotics are dangerous because their effective dosage ranges are narrow.³ Overdose is critical and leads to emergency department visits for uncontrolled bleeding; inadequate dosages lead to secondary (repeat), often fatal, thrombotic events. Dosages and half-lives differ among the antithrombotics because of variations in formulation and metabolism.^{4,5}

Because of these risks, laboratory monitoring or measurement of anticoagulant therapy is essential. Coagulation laboratory scientists and technicians perform countless prothrombin time (PT) assays, partial thromboplastin time (PTT, synonymous with activated partial thromboplastin time, APTT) assays, and chromogenic anti-factor Xa heparin assays to measure or monitor anticoagulant therapy; meanwhile, physicians and nurses regularly modify Coumadin and UFH dosages in response to laboratory outcomes. Although anticoagulant therapy measurement or monitoring may seem routine, vigilance is essential to provide consistently valid results in a dangerous therapeutic world.⁶

The antiplatelet drugs aspirin, clopidogrel, prasugrel, and ticagrelor, as well as LMWH, fondaparinux, and the direct oral anticoagulants (dabigatran, rivaroxaban, apixaban, and edoxaban), have fixed dose-response characteristics and do not require regular monitoring or laboratory-directed dose adjustment. However, though routine monitoring and dose adjustment may be unnecessary, these drugs require measurement in the conditions listed in **Box 43-1**.

COUMADIN THERAPY AND THE PROTHROMBIN TIME

Coumadin Is a Vitamin K Antagonist

As detailed in Chapter 37, the coagulation factors II (prothrombin), VII, IX, and X depend on vitamin K for normal production, as do coagulation control proteins C, S, and Z. Vitamin K is responsible for the

Table 43-1 Current Antithrombotics, Mode of Action, Measurement, Reversal

Antithrombotic	Indication	Mode of Action	Half-Life	Measurement	Reversal	FDA Cleared			
Coumadin	Prevent post-VTE rethrombosis, ischemic stroke	Oral VK antagonist	5 d	Monitor: PT/INR; CFX	Vitamin K, PCC, rFVIIa	1954			
UFH	Prevent post-VTE and ACS rethrombosis; intraoperative anticoagulation	IV AT activation, anti-IIa & anti-Xa	1–2 h	Monitor: PTT, anti-Xa, ACT	PS	1936			
LMWH	Prevent thrombosis after surgery, in medical conditions or in ACS; DVT/PE treatment	SC AT activation, anti-Xa	3–5 h	Anti-Xa	PS (partial)	1993			
Fondaparinux			12–17 h	Anti-Xa	None	2001			
Rivaroxaban	Prevent ischemic stroke, prevent thrombosis after orthopedic surgery, prevent post-VTE rethrombosis	Oral direct anti-Xa	12–15 h	PT?, anti-Xa	4-factor PCC, FEIBA, rFVIIa	2011			
Apixaban			12–15 h			2012			
Edoxaban			12 h			Clinical trials in progress			
Argatroban	Anticoagulation in HIT	IV DTI	50 min	PTT	Discontinue	1997			
Bivalirudin		IV DTI	25 min			Discontinue	2000		
Dabigatran	Prevent ischemic stroke	Oral DTI	17 h	PTT, ECT, ECA, DTT	None	2010			
Aspirin	Prevent acute coronary syndrome recurrence	Oral antiplatelet COX inhibitor		VerifyNow Aspirin, AspirinWorks, platelet aggregation	Discontinue	1900			
Clopidogrel						Oral, binds platelet P2Y ₁₂	VerifyNow P2Y	Discontinue	2000
Prasugrel						Oral, binds platelet P2Y ₁₂	VerifyNow P2Y	Discontinue	2009
Ticagrelor						Oral, binds platelet P2Y ₁₂	VerifyNow P2Y	Discontinue	2011
Eptifibatide						Maintain vascular patency during PCI and medical therapy for acute coronary syndrome	IV, binds platelet GP IIb/IIIa	2.5 h	VerifyNow GPI
Abciximab	GP IIb/IIIa	12–24 h	VerifyNow GPI	Discontinue	1993				
Tirofiban		2.5 h			1998				

ACS, acute coronary syndrome; ACT, activated clotting time; AT, antithrombin; CFX, chromogenic factor X activity; COX, cyclooxygenase; DTI, direct thrombin inhibitor; DTT, plasma-diluted thrombin time; ECA, ecarin chromogenic assay; ECT, ecarin clotting time; FEIBA, factor VIII inhibitor bypassing activity; GPI, glycoprotein inhibitor; INR, international normalized ratio; IV, intravenous; PCC, prothrombin complex concentrate; PT, prothrombin time; PTT, partial thromboplastin time; PS, protamine sulfate; rFVIIa, recombinant activated factor VII; SC, subcutaneous; VK, Vitamin K.

γ -carboxylation of a linear series of 12 to 18 glutamic acids near each molecule's N-terminus (amino terminus), a posttranslational modification that enables these coagulation factors and coagulation control proteins to bind ionic calcium (Ca^{2+}) and cell membrane phospholipids, especially phosphatidylserine (Figure 37-9). Vitamin K is concentrated in green tea, avocados, and green leafy vegetables and is produced by gut flora; its absence results in the production of nonfunctional *des- γ carboxyl* forms of factors II, VII, IX, and X and proteins C, S, and Z.

Coumadin (4-hydroxycoumarin, warfarin sodium) is a member of the coumarin drug family and is the formulation of coumarin most often used in North America.⁷ Another coumarin is dicoumarol (3,3'-methylenebis-[4-hydroxycoumarin]), the original anticoagulant extracted from moldy sweet clover, described in 1940, and used for many years as a rodenticide.⁸ Coumadin is a vitamin K antagonist that suppresses γ -carboxylation of glutamic acid by slowing the activity of the enzyme *vitamin K*

epoxide reductase (Figure 37-9). During Coumadin therapy, the activities of factors II, VII, IX, and X and proteins C, S, and Z become reduced as the nonfunctional des-carboxyl proteins are produced in their place. These are sometimes called *proteins induced by vitamin K antagonists* (PIVKAs); they bind few calcium ions, do not assemble on phospholipid surfaces with their substrates, and therefore do not participate in coagulation. Despite several developmental efforts, until 2009, Coumadin was the only oral anticoagulant in the United States, so Coumadin therapy has often been called *oral anticoagulant therapy* (OAT). The new direct-acting oral anticoagulants rivaroxaban, apixaban, edoxaban, and dabigatran have broadened the meaning of "OAT."

Coumadin Prophylaxis and Therapy

Physicians prescribe Coumadin prophylactically to prevent TIAs and strokes in patients with nonvalvular atrial fibrillation

BOX 43-1 Clinical Conditions That Require Measurement of Antiplatelet Drugs and Anticoagulants Besides Coumadin and UFH

- Renal disease: inadequate excretion, CrCl <30 mL/min
- Detection of noncompliance and underdosing
- Detection of comedication interference
- Acute hemorrhage (usually in emergency department or surgery)
 - Overdose, effects of comedication
 - Detection and identification; what anticoagulant is it?
 - Determine if reversal is working
- Bridging from one anticoagulant to another or discontinuing anticoagulant before surgery
- Resuming anticoagulant after surgery
- Unstable coagulation: pregnancy, liver disease, malignancy, chronic DIC
- Patients >75 years old (excluded from clinical trials)
- Patients with marginal fluid compartment (excluded from clinical trials)
 - >150 kg: proportionally reduced fluid compartment
 - <40 kg or pediatric: proportionally increased fluid compartment

and to prevent VTE after trauma, orthopedic surgery, and general surgery, and in a number of chronic medical conditions. They also prescribe Coumadin therapeutically to prevent DVT or PE recurrence. Coumadin is also used therapeutically after AMI if the event is complicated by congestive heart failure or coronary insufficiency and to control clotting in patients with mechanical heart valves. Coumadin is among the 20 most commonly prescribed drugs in North America.

Whether prescribed prophylactically or therapeutically, the standard Coumadin regimen begins with a 5-mg daily oral dose. The starting dosage for people over 70 and people who are debilitated, malnourished, or have congestive heart failure is 2 mg/day. For people simultaneously taking drugs that are known to raise Coumadin sensitivity, the starting dosage is 2 mg/day, and 2 mg/day is also the dosage used for those with inherited Coumadin sensitivity. There is no loading dose, and subsequent dosing is based on patient response as measured by the PT (next section). The activity of each of the vitamin K-dependent coagulation factors begins to decline immediately but at different rates (Figure 43-1), and it takes about 5 days for all the factors to reach therapeutic levels. Table 43-2 lists the plasma half-life, plasma concentration, and minimum effective plasma percentage of normal factor activity for the coagulation factors.

Control protein activities also become reduced, especially the activity of protein C, which has a 6-hour half-life, so for the first 2 or 3 days of Coumadin therapy the patient actually incurs the risk of thrombosis. For this reason, Coumadin therapy is “covered” by UFH, LMWH, or fondaparinux therapy for at least 5 days. Failure to provide anticoagulant therapy during this period may result in *warfarin skin necrosis*, a severe thrombotic reaction requiring débridement of dead tissue.⁹

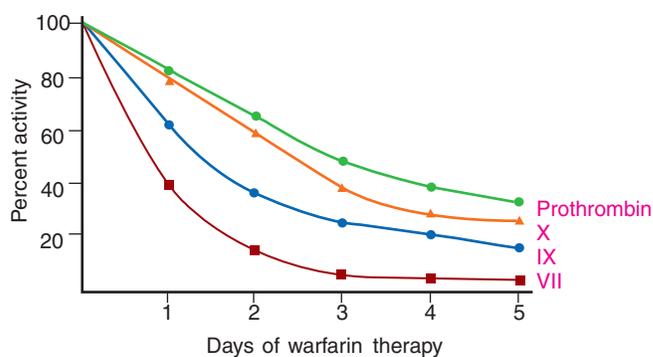


Figure 43-1 Factor VII activity decreases to 50% of normal 6 hours after Coumadin therapy is begun, prolonging the factor VIIa-sensitive prothrombin time to near the therapeutic INR of 2 to 3. The half-lives of factors II (prothrombin), IX, and X are longer than that of VII; factor II activity requires at least 3 days to decline by 50%. The patient gains full anticoagulation effects approximately 5 days after the start of Coumadin therapy.

TABLE 43-2 Plasma Half-Life, Normal Plasma Level, and Minimum Effective Hemostatic Level as a Percentage of Normal Level for the Coagulation Factors

Factor	Half-Life	Plasma Level	Hemostatic Level
Fibrinogen	4 days	280 mg/dL	50 mg/dL
Prothrombin	60 hr	1300 μ g/mL	20%
V	16 hr	680 μ g/mL	25%
VII	6 hr	120 μ g/mL	20%
VIII	12 hr	0.24 μ g/mL	30%
IX	24 hr	5 μ g/mL	30%
X	30 hr	1 mg/dL	25%
XI	2–3 days	6 μ g/mL	25%
XIII	7–10 days	290 μ g/mL	2%–3%
Von Willebrand factor	30 hr	6 μ g/mL	50%

Monitoring Coumadin Therapy Using the Prothrombin Time Assay

The PT effectively monitors Coumadin therapy because it is sensitive to reductions of factors II, VII, and X (Figure 43-2) (Chapter 42). The PT reagent consists of tissue factor, phospholipid, and ionic calcium, so it triggers the coagulation pathway at the level of factor VII. Owing to the 6-hour half-life of factor VII, the PT begins to prolong within 6 to 8 hours; however, anticoagulation becomes therapeutic only when the activities of factors II and X decrease to less than 50% of normal, which takes approximately 5 days.

The first PT is collected and performed 24 hours after therapy is initiated; subsequent PTs are performed daily until at least two consecutive results are within the target therapeutic range. Monitoring continues every 4 to 12 weeks until the completion of therapy, which often lasts for 6 months following a thrombotic event.¹⁰ Coumadin therapy for stroke prevention in atrial fibrillation is indefinite, possibly lifelong. Because the therapeutic range is narrow, close monitoring is essential for successful Coumadin therapy. Under-anticoagulation signals

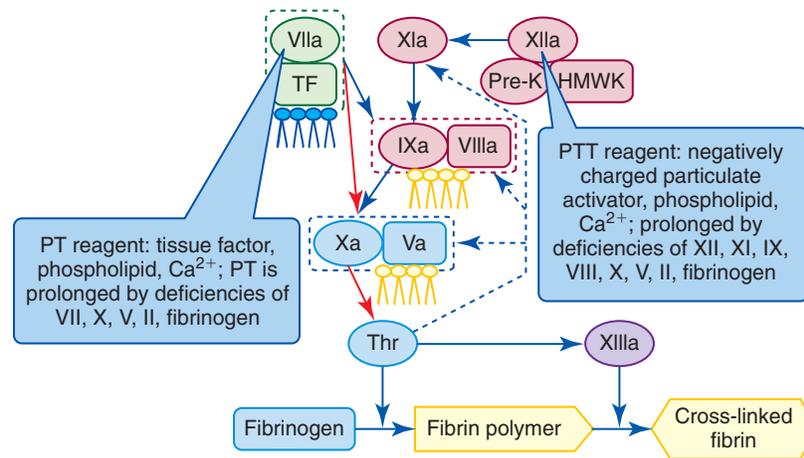


Figure 43-2 The prothrombin time (PT) reagent activates the extrinsic coagulation pathway beginning with factor VII. The PT is prolonged by deficiencies of factors VII, X, V, II (prothrombin), and fibrinogen when the fibrinogen concentration is less than 100 mg/dL. The PT is prolonged in Coumadin therapy because it responds to the reduced VII, X, and prothrombin activity. The partial thromboplastin time (PTT) reagent activates the intrinsic coagulation pathway through factor XII, in association with prekallikrein (pre-K) and high-molecular-weight kininogen (HMWK). The PTT is prolonged by deficiencies of pre-K; HMWK; factors XII, XI, IX, VIII, and X; prothrombin; and fibrinogen when the fibrinogen concentration is less than 100 mg/dL. The PTT is prolonged in unfractionated heparin (UFH) therapy because UFH activates the plasma control protein antithrombin, which neutralizes the serine proteases XIIa, XIa, Xa, IXa, and IIa (thrombin [Thr]). The PTT is also prolonged by lupus anticoagulant. *TF*, Tissue factor.

the danger of thrombosis or secondary thrombosis (rethrombosis); overdose carries the danger of hemorrhage.

Reporting Prothrombin Time Results and the International Normalized Ratio

The medical laboratory technician or scientist reports PT results to the nearest tenth of a second and provides the PT reference interval in seconds for comparison. In view of the inherent variations among thromboplastin reagents and to accomplish interlaboratory normalization, all laboratories report the international normalized ratio (INR) for patients who have reached a stable response to Coumadin therapy. Laboratory practitioners use the following formula:¹¹

$$\text{INR} = (PT_{\text{patient}}/PT_{\text{normal}})^{\text{ISI}}$$

where PT_{patient} is the PT of the patient in seconds, PT_{normal} is the geometric mean of the PT reference interval in seconds, and ISI is the international sensitivity index applied as an exponent.

Thromboplastin producers generate the ISI by performing an orthogonal regression analysis comparing the results of their PT reagents for 50 or more Coumadin plasma specimens and 10 or more normal specimens with the results of the *international reference thromboplastin* (World Health Organization human brain thromboplastin) on the same plasmas.¹² Most manufacturers provide ISIs for a variety of coagulation instruments, because each coagulometer may respond differently to their thromboplastins; for instance, some coagulometers rely on photometric plasma changes, whereas others use an electromechanical system (Chapter 44). Most thromboplastin reagents have ISIs near 1.0, matching the ISI of the World Health Organization's international reference thromboplastin. Automated coagulometers "request" the reagent ISI from the operator or obtain it electronically from a reagent vial label bar code, and compute the

INR for each assay result. Although INRs are meant to be computed only for patients in whom the response to Coumadin has stabilized, they typically are reported for all patients, even those who are not taking Coumadin. During the first 5 days of Coumadin therapy, the astute physician and medical laboratory practitioner ignore the INR as unreliable and interpret the PT results in seconds, comparing it with the reference interval.

Coumadin International Normalized Ratio Therapeutic Range

The physician adjusts the Coumadin dosage to achieve the desired INR of 2 to 3, or 2.5 to 3.5 if the patient has a mechanical heart valve. INRs greater than 4 are associated with increased risk of hemorrhage and require immediate communication with the clinician who is managing the patient's case.¹³ Dosage adjustments are made conservatively because the INR requires 4 to 7 days to stabilize, but an elevated INR accompanied by the symptoms of anatomic bleeding is a medical emergency.

Monitoring Coumadin Therapy Using the Chromogenic Factor X Assay

The chromogenic coagulation factor X assay (not to be confused with the chromogenic anti-factor Xa heparin assay) may be used as an alternative to the PT/INR system, eliminating the necessity for normalization.¹⁴ The therapeutic range is determined locally by comparison to the INR and typically is close to 20% to 40% of normal factor X activity.¹⁵ The chromogenic factor X assay is useful when the PT is compromised by lupus anticoagulant, a factor inhibitor, or a coagulation factor deficiency.¹⁶

Effect of Diet and Drugs on Coumadin Therapy

Dietary vitamin K decreases Coumadin's effectiveness and reduces the INR. Green vegetables are an important source of

vitamin K, but vitamin K also is concentrated in green tea, cauliflower, liver, avocados, parenteral nutrition formulations, multivitamins, red wine, over-the-counter nutrition drinks, and over-the-counter dietary supplements. A patient who is taking Coumadin is counseled to maintain a regular balanced diet, avoid supplements, and to follow up dietary changes or dietary supplement changes with additional PT assays and dosage adjustments, if indicated.

Coumadin is metabolized in the mitochondrial cytochrome P-450 (CYP 2C9) pathway of hepatocytes—the “disposal system” for at least 80 drugs. Theoretically, use of any drug metabolized through the CYP 2C9 pathway may unpredictably suppress or enhance the effects of Coumadin. Amiodarone, metronidazole, and cimetidine typically double or triple the INR. Any change in drug therapy, like a change in diet, must be followed up with additional PT assays and dosage adjustments.

Coumadin is contraindicated during pregnancy because it causes birth defects. When anticoagulation is desired during pregnancy—for instance, in women who possess a thrombosis risk factor—LMWH or fondaparinux is prescribed. There are no current recommendations for the direct oral anticoagulants during pregnancy.

Effect of Polymorphisms on Coumadin Therapy

Two genetic polymorphisms generate variations in enzymes of the cytochrome P-450 pathway. These are *CYP2C9*2* and *CYP2C9*3*, which reduce enzyme pathway activity and slow the metabolic breakdown of Coumadin. Likewise, there is a polymorphism that affects the key enzyme of vitamin K metabolism, *vitamin K epoxide reductase*. This polymorphism, named *VKORC1*, slows vitamin K reduction, which makes the patient more sensitive to Coumadin.¹⁷ In patients possessing one, two, or all three of these polymorphisms, Coumadin therapy should begin at 2 mg/day and should be adjusted and monitored daily until the INR remains consistently in the therapeutic range. The standard 5 mg/day regimen risks hemorrhage in patients who possess these polymorphisms. In 2007, the FDA required that drug manufacturers add a statement on all vials of Coumadin recommending that physicians screen patients for these common dosage-affecting polymorphisms. Although the FDA recommendation does not carry the weight of a black box warning issued by the FDA for drug use, numerous molecular diagnostics manufacturers have developed short turnaround assays for these three polymorphisms. Screening for these polymorphisms is the first and most public example of pharmacogenomic laboratory testing, although not universally endorsed.¹⁸

Conversely, *Coumadin receptor insufficiency* may render the patient resistant to Coumadin therapy. Some patients require dosages of 20 mg/day or higher to achieve a therapeutic INR. The search is on for polymorphisms of the vitamin K reductase pathway responsible for “Coumadin resistance.”¹⁹

Effect of Direct Thrombin Inhibitors on the Prothrombin Time

The intravenously administered DTIs argatroban and bivalirudin, which are used in place of heparin as a life-saving measure

TABLE 43-3 Recommendations for the Reversal of Coumadin Overdose Based on International Normalized Ratio (INR) and Bleeding

Bleeding	INR	Intervention
No significant bleeding	3–5	Reduce dosage or omit one dose, monitor INR frequently
	5–9	Omit Coumadin, monitor INR frequently, consider oral vitamin K (≤ 5 mg) if high risk for bleeding (surgery)
	>9	Stop Coumadin, give 5–10 mg oral vitamin K, monitor INR frequently
Serious bleeding	Any INR	Stop Coumadin; give 10 mg vitamin K by intravenous push, may repeat every 12 hr; give thawed fresh-frozen plasma, prothrombin complex concentrate, or recombinant factor VIIa
Life-threatening bleeding	Any INR	Same as for serious bleeding, except stronger indication for recombinant factor VIIa

for patients with HIT, and the anti-factor Xa direct oral anticoagulants rivaroxaban and apixaban, may prolong the PT (depending on the PT reagent). In switching to Coumadin therapy, the combination of a DTI or direct oral anticoagulant and Coumadin can nearly double the PT for the duration of action of the DTI or direct oral anticoagulant, which may extend 3 or 4 days.²⁰ The chromogenic factor X assay is an effective means for monitoring Coumadin dosage during the crossover period.

Reversing Bleeding Caused by a Coumadin Overdose

Table 43-3 provides recommendations for the reversal of a Coumadin overdose based on INR and clinical evidence of bleeding. Reversal requires oral or intravenous vitamin K and, if bleeding is severe, a means for substituting active coagulation factors such as fresh-frozen plasma, recombinant activated factor VII (NovoSeven, Novo Nordisk, Princeton, NJ), activated three-factor prothrombin complex concentrate (FEIBA FH, Baxter Healthcare Corporation, Westlake Village, CA), three-factor prothrombin complex concentrate (“non-activated” Profilnine SD; Grifols Biologicals, Inc., Los Angeles, CA), or four-factor prothrombin complex concentrate (4F-PCC, Kcentra; CSL Behring, King of Prussia, PA).²¹

UNFRACTIONATED HEPARIN THERAPY AND THE PARTIAL THROMBOPLASTIN TIME

Heparin Is a Catalyst That Activates Antithrombin to Neutralize Serine Proteases

Standard UFH is a biological substance, first described in 1916. It is a mixture of sulfated glycosaminoglycans (polysaccharides) extracted from porcine mucosa. The molecular weight of UFH ranges from 3000 to 30,000 Daltons (average molecular weight 15,000 Daltons). Approximately one third of its molecules support somewhere on their length a high-affinity pentasaccharide that binds plasma antithrombin. The anticoagulant

action of UFH is indirect and catalytic, relying on antithrombin. The pentasaccharide-bound antithrombin undergoes a steric change (allostery), exposing an anticoagulant site that covalently binds and inactivates the coagulation pathway serine proteases, factors IIa (thrombin), IXa, Xa, XIa, and XIIa (Chapter 37). Laboratory practitioners call activated antithrombin a *serine protease inhibitor* (SERPIN), and the protease-binding reaction yields, among other products, the measurable inactive plasma complex thrombin-antithrombin (TAT).

Heparin supports the thrombin-antithrombin reaction through a “bridging” mechanism (Figure 43-3). If the heparin molecule exceeds 17 linear saccharide units, thrombin assembles on the heparin molecule near the activated antithrombin. Bridging drives the thrombin-antithrombin reaction at a rate four times that of the factor Xa-antithrombin reaction, because factor Xa becomes inactivated only through antithrombin’s steric modification, and its covalent binding is not enhanced by bridging.

UFH preparations vary in average molecular weight, molecule length, and efficacy. Individual patient heparin dose-responses diverge markedly, because numerous plasma and cellular proteins bind UFH at varying rates. Consequently, laboratory monitoring is essential.²²

Unfractionated Heparin Therapy

Physicians administer UFH intravenously to treat VTE, to provide initial treatment of AMI, to prevent reocclusion after stent placement, and to maintain vascular patency during cardiac surgery using cardiopulmonary bypass (CPB) with extracorporeal circulation. Different dosing regimens are used in various settings. For VTE treatment, therapy begins with a bolus of 5000 to 10,000 units, followed by continuous infusion at approximately 1300 units/hour, adjusted to patient weight. UFH therapy is discontinued when the acute clinical state has

resolved or after the procedure or surgery. If necessary the patient will be switched to a non-intravenous anticoagulant to prevent future thrombotic events. To avoid HIT (Chapters 39 and 40), LMWH or other anticoagulants are used in place of UFH where possible.

Monitoring Unfractionated Heparin Therapy Using the Partial Thromboplastin Time

Because of its inherent pharmacologic variations and narrow therapeutic range, UFH therapy is diligently monitored using the PTT (Figure 43-2). Blood is collected and assayed before therapy is begun to ensure that the baseline PTT is normal.²³ A prolonged baseline PTT may indicate the presence of a lupus anticoagulant, factor inhibitor, or a factor deficiency and confuses the therapeutic interpretation. In such cases, the laboratory practitioner switches to the chromogenic anti-factor Xa heparin assay throughout the duration of therapy (discussed later in the chapter).

A second specimen is collected at least 4 to 6 hours but not longer than 24 hours after the initial bolus and a PTT is measured on this specimen. The PTT becomes prolonged within minutes of UFH administration, which reflects the immediate anticoagulation effect of UFH. The result for this specimen should fall within the therapeutic range, which is established by the laboratory practitioner (next section) and reported with the result. The physician or nurse adjusts the infusion rate to ensure that the PTT result is within the target range. PTT measurement is subsequently repeated every 24 hours, and the dosage is continually readjusted until UFH anticoagulation is complete. The physician also monitors the platelet count daily. A 40% or greater reduction in the platelet count, even within the reference interval, is evidence for HIT (see the discussion of the “4Ts” HIT diagnosis system in Chapter 39). If HIT is suspected, UFH therapy is immediately discontinued and replaced with DTI therapy.

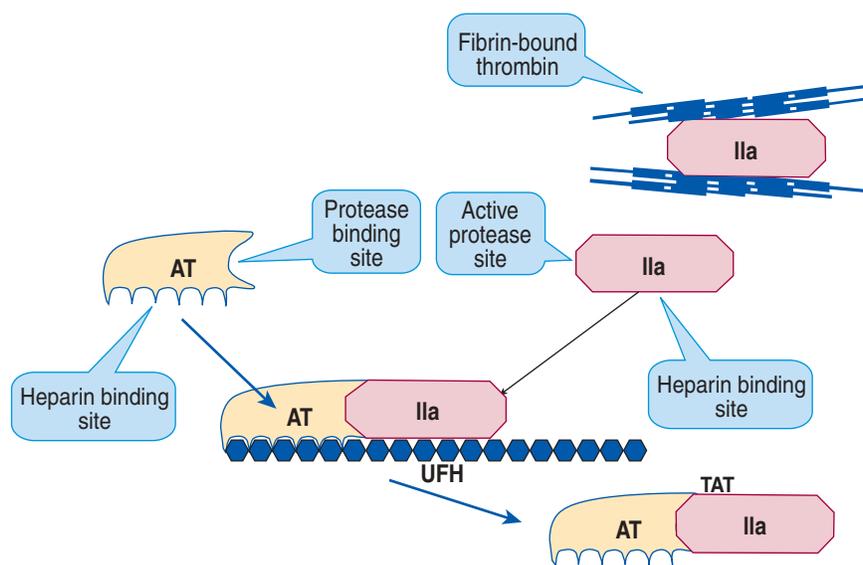


Figure 43-3 The heparin binding site of antithrombin (AT) binds a specific pentasaccharide, producing an allosteric change that activates AT. Factor IIa (thrombin) assembles on the heparin surface, provided the molecule is at least 17 saccharide units long. AT binds factor IIa, and the complex is released from unfractionated heparin (UFH) to form the soluble, measurable thrombin-antithrombin (TAT) complex. The UFH recycles. Fibrin-bound IIa does not enter the reaction.

Determining the Partial Thromboplastin Time Therapeutic Range for Unfractionated Heparin Therapy

The hemostasis laboratory is required to establish and communicate a PTT therapeutic range to monitor and manage UFH therapy. The medical laboratory technician or scientist collects 20 to 30 plasma specimens from patients being infused with UFH at all levels of anticoagulation, ensuring that fewer than 10% of the specimens are collected from the same patient, and measures PTT for all.²⁴ The specimens must be from patients who are not receiving simultaneous Coumadin therapy; that is, their PT results must be normal. Chromogenic anti-factor Xa heparin assays are performed on all specimens, plus at least 10 specimens from healthy normal subjects, and the paired results are displayed on a linear graph (Figure 43-4). The range in seconds of PTT results that corresponds to 0.3 to 0.7 chromogenic anti-factor Xa heparin units/mL is the therapeutic range.²⁵ This is known as the *ex vivo* or *Brill-Edwards method* for establishing the heparin therapeutic range of the PTT, and its use is required by laboratory certification and licensing agencies. Other approaches to determining the PTT therapeutic range for UFH therapy are discouraged. For instance, experts once recommended that the PTT therapeutic range be established as 1.5 to 2.5 times the mean of the reference interval. This approach, however, must be avoided as it consistently results in under-anticoagulation, which raises the risk of a secondary thrombotic event. In addition, the practice of developing a therapeutic range by “spiking” normal plasma with measured volumes of heparin is prohibited because the curve that is generated tends to flatten at higher concentrations.²⁶

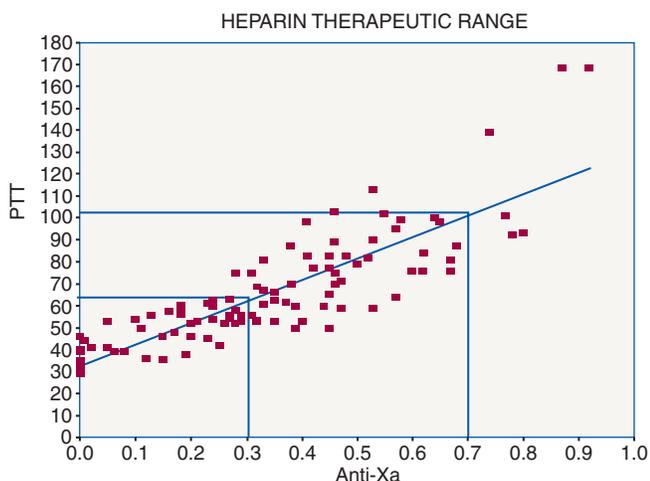


Figure 43-4 Laboratory scientists establish the partial thromboplastin time (PTT) therapeutic range for unfractionated heparin (UFH) by collecting specimens from 20 to 30 patients receiving UFH at representative dosages who have normal PTs and at least 10 individuals not receiving heparin. PTT and chromogenic anti-factor Xa heparin assays are performed on all specimens, and a linear graph of paired results is prepared with PTT on the vertical scale. The PTT range in seconds is correlated with the chromogenic anti-factor Xa therapeutic range of 0.3 to 0.7 units/mL or the prophylactic range of 0.1 to 0.4 units/mL.

Clinical Utility of Monitoring Unfractionated Heparin Therapy Using the Partial Thromboplastin Time

The medical laboratory practitioner reports the PTT results, the reference interval, and the UFH therapeutic range to the clinician (physician, nurse, or pharmacist) who is managing the patient's UFH dosage. Because reagent sensitivity varies among producers and among individual producers' reagent lots, the clinician must evaluate PTT results in relation to the institution's current published therapeutic range and reference interval.²⁷ No system analogous to the INR exists for normalizing PTT results, because reagents and patient responses are too variable.²⁸ While the PTT is used most often to measure the effects of UFH therapy, LMWH (next section) selectively catalyzes the neutralization of factor Xa more avidly than the neutralization of thrombin, thus its effects cannot be measured using the PTT. However, the chromogenic anti-factor Xa heparin assay may be used to assay UFH, LMWH, and fondaparinux.

Limitations of Monitoring Unfractionated Heparin Therapy Using the Partial Thromboplastin Time

Several conditions render the patient unresponsive to heparin therapy, a circumstance called *heparin resistance*.²⁹ Inflammation typically is accompanied by fibrinogen levels raised to greater than 500 mg/dL and coagulation factor VIII activities of greater than 190% above the mean of the reference interval. Both elevations render the PTT less sensitive to the effects of heparin. Further, in many patients, antithrombin activity becomes depleted as a result of prolonged therapy or an underlying deficiency secondary to chronic inflammation. In this instance, the PTT result remains below the therapeutic range, becoming only modestly prolonged despite ever-increasing heparin dosages. Inflammation may be reduced through administration of steroids and aspirin or nonsteroidal anti-inflammatory drugs, and antithrombin concentrate may be administered. In the interim, however, it is necessary to use an alternative assay such as the chromogenic anti-factor Xa heparin assay.

Platelets in anticoagulated whole-blood specimens release platelet factor 4, a heparin-neutralizing protein (Chapter 13). In specimens from patients receiving heparin therapy, the PTT begins to shorten as soon as 1 hour after collection because of in vitro platelet factor 4 release unless the specimen is centrifuged and the platelet-poor plasma is removed from the cells (Chapter 42).³⁰ Hypofibrinogenemia, factor deficiencies, and the presence of lupus anticoagulant, fibrin degradation products, or paraproteins prolong the PTT independent of heparin levels.³¹

Monitoring Unfractionated Heparin Therapy Using the Activated Clotting Time

The ACT is a 1966 modification of the time-honored but obsolete Lee-White whole blood clotting time test. The ACT is a popular point of care assay that is used in clinics, at the inpatient's bedside, in the cardiac catheterization laboratory, or

in the surgical suite, and it is particularly useful at the high UFH dosages, 1 to 2 units/mL, used in percutaneous intervention (PCI, cardiac catheterization) and in cardiac surgery using extracorporeal circulation.³²

ACT assay distributors such as International Technidyne Corporation, Edison, NJ, the makers of the Hemochron Response (Chapter 44), provide evacuated blood specimen collection tubes that contain 12 mg of diatomaceous earth, a particulate clot activator. The negative pressure within the tube is calibrated to collect 2 mL of blood. As soon as the specimen is collected, the tube is placed in the instrument cuvette well, where it is rotated and continuously monitored. When a clot forms, a magnet positioned within the sample is pulled away from a sensing device, which stops the timer. The time interval to clot formation is recorded automatically. The results of the ACT assay are comparable to those of the PTT assay for UFH monitoring, provided adequate quality control steps are taken. The median of the ACT reference interval is 98 seconds. Heparin is administered to yield results of 200 to 240 seconds in PCI or 400 to 450 seconds during cardiac surgery, levels at which the PTT is ineffective.

Reversal of Unfractionated Heparin Overdose Using Protamine Sulfate

At completion of cardiac surgery when the extracorporeal circuit is to be terminated, heparin anticoagulation needs to be quickly reversed. In other settings, a UFH overdose, or co-administration with aspirin, fibrinolytic therapy, or a GPI, may raise the risk of bleeding. Protamine sulfate, a cationic protein extracted from salmon sperm, neutralizes UFH at a ratio of 100 units of heparin per milligram of protamine sulfate. The health care provider administers protamine sulfate slowly by intravenous push. The effect of the protamine sulfate may be detected by the shortening of the PTT or ACT. Protamine sulfate also neutralizes LMWH, although the neutralization is incompletely reflected in the results of the chromogenic anti-factor Xa heparin assay described in the following paragraphs.

Protamine sulfate has also been implicated as causing a delayed form of HIT, consequently, platelet counts for patients who have received protamine sulfate are routinely monitored.^{33,34}

LOW-MOLECULAR-WEIGHT HEPARIN THERAPY AND THE CHROMOGENIC ANTI-FACTOR Xa HEPARIN ASSAY

Low-Molecular-Weight Heparin Is Produced from Unfractionated Heparin

Uncertainty about UFH dose response and the ever-present threat of HIT led to the development of LMWH, which was cleared for anticoagulant prophylaxis in the United States and Canada in 1993.³⁵ LMWH is prepared from UFH using chemical (enoxaparin, Lovenox, Sanofi-Aventis, Bridgewater, NJ) or enzymatic fractionation (tinzaparin sodium, Innohep, LEO Pharmaceutical Products, Ballerup, Denmark).³⁶ Fractionation yields a product with a mean molecular weight of 4500 to 5000 Daltons, about one third the mass of UFH. LMWH possesses the same active pentasaccharide sequence as UFH; however, the overall shorter polysaccharide chains provide little space for thrombin bridging, so the thrombin neutralization response is reduced (Figure 43-5). The factor Xa neutralization response is unchanged, however, because this reaction does not rely on factor Xa binding to heparin's polysaccharide chain, so LMWH provides nearly the same anticoagulant efficacy as UFH, although predominantly through factor Xa inhibition.

LMWH is administered by subcutaneous injection once or twice a day using premeasured syringes at selected dosages—for instance, 30 mg subcutaneously every 12 hours or 40 mg subcutaneously once daily. Prophylactic applications provide coverage during or after general and orthopedic surgery and trauma, typically for 14 days from the time of the event. LMWH also is used to treat DVT, PE, and unstable angina. LMWH is indicated during pregnancy for women at risk of VTE, because Coumadin, which causes birth defects, cannot be used. When patients who are taking Coumadin require surgery

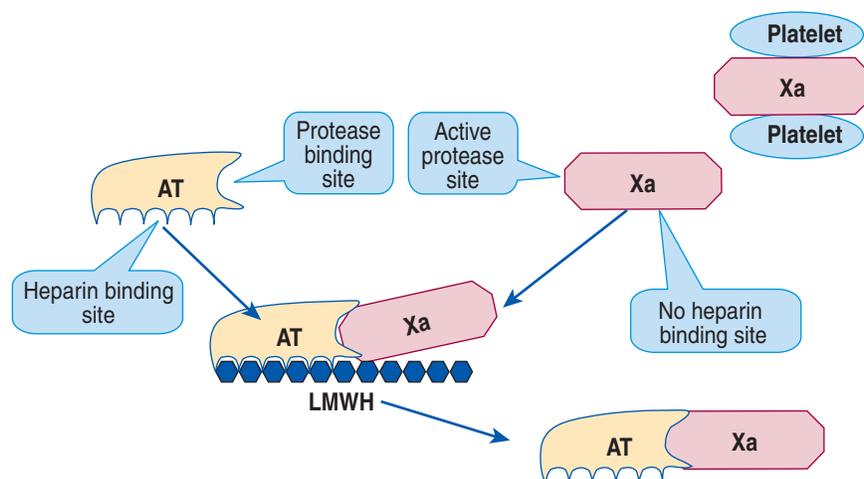


Figure 43-5 The antithrombin (AT) binding site binds a specific heparin pentasaccharide, producing an allosteric change that activates AT. The low-molecular-weight heparin (LMWH) molecule is too short to support a factor IIa-AT reaction; however, the activated AT binds factor Xa independently of the bridging phenomenon, producing a soluble AT-factor Xa complex. The LMWH recycles.

it is discontinued for up to a week before the procedure and replaced with LMWH.³⁷

The advantages of LMWH are rapid bioavailability after subcutaneous injection, making intravenous administration unnecessary; a half-life of 3 to 5 hours compared with 60 to 90 minutes for UFH; and a fixed dose response that eliminates the need for laboratory monitoring, although laboratory measurement is still required in the conditions listed in [Box 43-1](#). The risk of HIT is reduced by 90% in people who have never received heparin before; however, LMWH may cross-react with previously formed antibodies against heparin-platelet factor 4. Consequently, LMWH is contraindicated in patients who developed HIT after UFH therapy. The risk of LMWH-induced bleeding is less than that for UFH.

Measuring Low-Molecular-Weight Heparin Therapy

The kidneys alone clear LMWH, so it accumulates in renal insufficiency. Laboratory measurement of LMWH therapy is necessary when the creatinine clearance is less than 30 mL/min or the serum creatinine is greater than 4 mg/dL. During LMWH therapy, creatinine assays are performed periodically to document kidney function and avoid the risk of LMWH accumulation in plasma. LMWH therapy in children, adults under 50 kg, adults over 150 kg, and during pregnancy also requires measurement because of fluid compartment imbalances or unstable coagulation ([Box 43-1](#)).

The phlebotomist collects a blood specimen 4 hours after subcutaneous injection, and the plasma is tested using the chromogenic anti-factor Xa heparin assay. The PTT is insensitive to LMWH. The chromogenic anti-factor Xa heparin assay employs a reagent that provides a fixed concentration of factor Xa and substrate specific to factor Xa ([Figure 43-6](#)). Some distributors add fixed concentrations of antithrombin, and others none; the latter rely on the patient's plasma antithrombin and provide sensitivity to antithrombin depletion or deficiency. Heparin forms a complex with reagent factor Xa and antithrombin; a measured excess of factor Xa digests the substrate,

yielding a colored product whose intensity is inversely proportional to heparin concentration.

To prepare a standard curve, the laboratory practitioner obtains the characteristic UFH or LMWH calibrators from distributors, then computes and prepares dilutions that “bracket” the reference and the therapeutic range. If the chromogenic anti-factor Xa heparin assay is to be used to monitor UFH and LMWH, a single hybrid standard curve may be prepared.^{38,39} A separate curve is necessary to monitor the pentasaccharide fondaparinux (next section). The prophylactic range for LMWH is 0.2 to 0.5 unit/mL, and the therapeutic range is 0.5 to 1.2 units/mL.²⁴

The chromogenic anti-factor Xa heparin assay is the primary assay available to measure LMWH and fondaparinux. It may also be used in place of the PTT to assay UFH with little or no modification, and it substitutes for the PTT when clinical or laboratory conditions render PTT results unreliable. The chromogenic anti-factor Xa heparin assay is “tertiary” in the sense that it measures the heparin concentration and not heparin's anticoagulant effects; however, the assay is precise and, in contrast to the PTT, is affected by few interferences. The chromogenic anti-factor Xa heparin assay is also the reference method for establishing the PTT therapeutic range. Laboratory directors have begun to recognize the merits of the chromogenic anti-factor Xa heparin assay and substitute it for the PTT in monitoring all UFH therapy, as well as in measuring therapy levels of LMWH and fondaparinux.

MEASURING PENTASACCHARIDE THERAPY USING THE CHROMOGENIC ANTI-FACTOR Xa HEPARIN ASSAY

Fondaparinux sodium (Arixtra; GlaxoSmithKline, Research Triangle Park, NC) is a synthetic formulation of the active pentasaccharide sequence in UFH and LMWH ([Figure 43-7](#)). Fondaparinux raises antithrombin activity 400-fold.⁴⁰ It is equivalent in clinical efficacy to LMWH with a reduced major bleeding effect and has a reproducible dose response and a

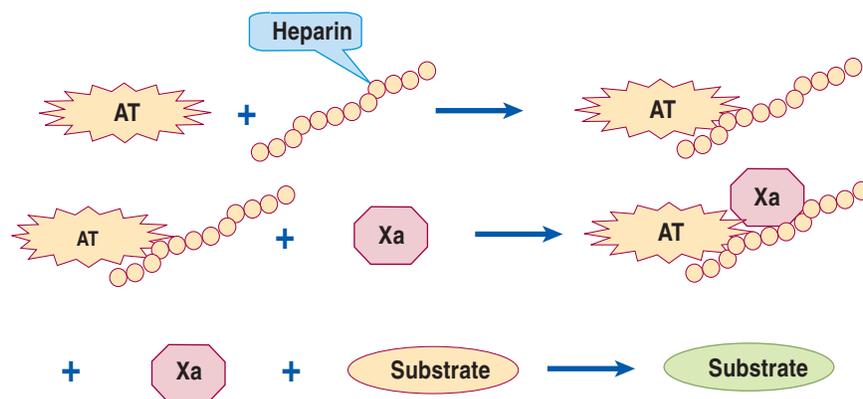


Figure 43-6 The chromogenic anti-factor Xa heparin assay. The reagent is a mixture of antithrombin (AT) and a measured excess of Xa. Most kits do not provide AT and rely solely on patient plasma AT. AT binds heparin, and this complex binds factor Xa. Excess free factor Xa digests its substrate to produce a colored end product. The color intensity of the product is inversely proportional to plasma heparin. This assay is used for unfractionated heparin, low-molecular-weight heparin, and pentasaccharide fondaparinux; a dedicated standard curve is required for fondaparinux.

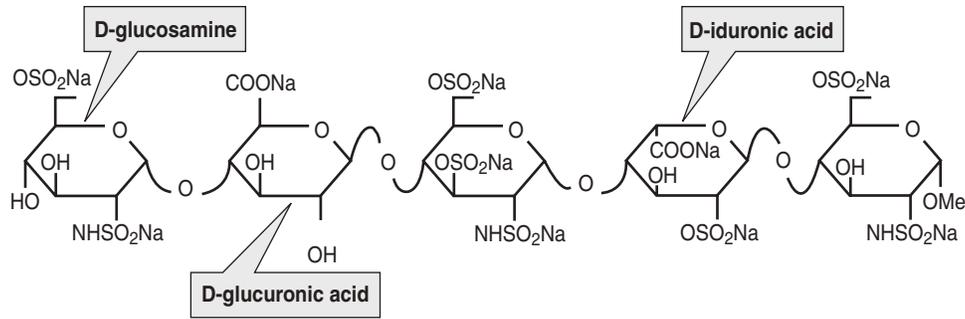


Figure 43-7 The specific saccharide sequence in unfractionated heparin and low-molecular-weight heparin (glucosamine, glucuronic acid, glucosamine, iduronic acid, glucosamine) is synthesized to make fondaparinux, which binds and activates the heparin-binding site of antithrombin. (From Turpie AGG: Pentasaccharides. *Semin Hematol* 39:159-171, 2002.)

desirable half-life of 17 to 21 hours. Because of the extended half-life, fondaparinux is administered in once-a-day subcutaneous injections of 2.5 to 7.5 mg.⁴¹ Fondaparinux is FDA-cleared for prevention of VTE after orthopedic and abdominal surgery and for treatment of acute VTE events, but its use is contraindicated in patients with creatinine clearance values of less than 30 mL/min.⁴²

The chromogenic anti-factor Xa heparin assay is used to measure fondaparinux therapy in children, adults below 50 kg or over 150 kg, patients receiving treatment for more than 7 to 8 days, and pregnant women.⁴³⁻⁴⁵ Blood is collected 4 hours after injection, and the target range, derived from clinical studies, though not confirmed by outcome studies, is 0.2 to 0.4 mg/mL for a 2.5 mg dose and 0.5 to 1.5 mg/mL for a 7.5 mg dose. The operator prepares a calibration curve using fondaparinux—not UFH, LMWH, or a hybrid calibrator—because concentrations are expressed in mg/mL, not units per mL. The PTT is not sensitive to the effects of fondaparinux because, although fondaparinux reacts with antithrombin and factor Xa, it does not inhibit thrombin or factors IXa, XIa, or XIIa.

In the event of bleeding associated with fondaparinux overdose, protamine sulfate is ineffective. Recombinant activated factor VII (rFVIIa, NovoSeven; Novo Nordisk, Princeton, NJ) may partially reverse the effects of fondaparinux.⁴⁶

MEASURING ORAL DIRECT FACTOR Xa INHIBITORS

Oral rivaroxaban (Xarelto; Bayer Healthcare AG, Leverkusen, Germany; Janssen Pharmaceuticals, Inc., Raritan, NJ) is an oxazolidinone derivative that directly and stoichiometrically inhibits factor Xa.^{47,48} It inhibits free factor Xa, factor Xa that is bound by factor IXa, and clot-bound factor Xa.⁴⁵ As established by results of several clinical trials, rivaroxaban has efficacy and safety characteristics equivalent to LMWH or Coumadin.

Rivaroxaban was cleared in September 2008 by both Health Canada and the European Medicines Agency and in July 2011 by the U.S. FDA for VTE prophylaxis in patients who are undergoing total knee or total hip replacement surgery. The FDA also cleared rivaroxaban in November 2011 for prevention of ischemic stroke in patients with chronic nonvalvular atrial fibrillation at 20 mg/day and for prevention of secondary thrombosis

subsequent to DVT or PE at 15 mg twice a day in December 2012.^{49,50} The European Medicines Agency cleared rivaroxaban at 2.5 mg/day for prevention of secondary thrombosis following acute myocardial infarction in March 2013, but that same month the FDA deferred clearance for the same indication having considered a dosage of 10 mg/day.^{47,51} The distributor re-filed in August 2013. As of June 2014, rivaroxaban has not been approved for use in patients with MI.

Rivaroxaban slightly prolongs the PT, as reported in several clinical trials, but to a lesser extent than Coumadin. Attempts to correlate PT results with dosage have revealed variability among PT thromboplastin reagents, rendering the PT only partially valid as a means to measure rivaroxaban.⁵²⁻⁵⁴ Rivaroxaban may also be assayed using a version of the chromogenic anti-factor Xa heparin assay. The assay must be calibrated using rivaroxaban in place of UFH, LMWH, or fondaparinux.⁵⁵ Using either PT or anti-factor Xa, laboratory practitioners are working to correlate laboratory results with clinical outcomes in an effort to provide a therapeutic range.⁵⁶

Like rivaroxaban, oral apixaban (Eliquis; Pfizer, New York, NY; Bristol-Myers Squibb, New York, NY) is a small oxazolidinone-derived direct stoichiometric factor Xa-inhibiting anticoagulant. The results of clinical trials reveal that apixaban actually improves on the efficacy and safety of Coumadin. The FDA cleared apixaban in December 2012 for the prevention of ischemic stroke in atrial fibrillation. The dosage for this indication is 2.5 mg twice a day. Apixaban has a weaker effect on the PT than rivaroxaban and Coumadin but may be measured using the PT, provided the operator first determines the sensitivity of the reagent. The chromogenic anti-factor Xa may also be used to measure apixaban when controls and calibrators become available in the North American market.⁵⁷

A third oral direct-acting anti-factor Xa anticoagulant, edoxaban (Lixiana; Daiichi-Sankyo, Tokyo, Japan), cleared in Japan in July 2011 for clot prevention in patients who have had total knee or hip replacement, is currently in phase III trials worldwide. Its characteristics mirror rivaroxaban and apixaban, and its plasma concentration may likely be measured using the same methods, but this has not yet been determined.⁵⁸

The oral direct anti-factor Xa inhibitors rivaroxaban, apixaban, and edoxaban all possess half-lives of approximately 12 hours. Apixaban may have an advantage in that 70% of the active drug is cleared by the liver and only 30% by the kidney, so

dosage is relatively unaffected by renal insufficiency. There is no current recommended reversal agent for hemorrhages caused by overdoses of these drugs; however, some clinicians report normalization of laboratory test results by the use of factor eight inhibitor bypassing agent (FEIBA; Baxter, Deerfield, IL), four-factor (II, VII, IX, and X) prothrombin complex concentrate (Kcentra; DSL Behring, King of Prussia, PA), or recombinant activated factor FVII (rFVIIa, NovoSeven; Novo Nordisk, Princeton, NJ). All oral direct factor Xa inhibitors are prescribed with no monitoring (contrary to Coumadin); however, the clinical conditions listed in [Box 43-1](#) may dictate the need to measure drug levels at specific times. A drug-specific chromogenic anti-factor Xa assay, utilizing a sodium citrate plasma sample, will likely be the assay of choice in most laboratories. Although the anti-factor Xa assay has been used for several years to monitor UFH and measure LMWH and fondaparinux, it awaits FDA clearance and is currently classified as *research use only* when applied to measuring rivaroxaban, apixaban, and edoxaban.

DIRECT THROMBIN INHIBITORS

Argatroban

The intravenous use DTIs argatroban and bivalirudin reversibly bind and inactivate free and clot-bound thrombin ([Figure 43-8](#)). DTIs are substituted for UFH or LMWH when HIT is suspected or confirmed using the “4Ts” assessment system (Chapter 39). Without the use of an intravenous DTI, the risk of thrombosis is 50% for 30 days after heparin is discontinued. In HIT, Coumadin, UFH, and LMWH are contraindicated.

Argatroban (Novostan; GlaxoSmithKline, Research Triangle Park, NC) is a non-protein L-arginine derivative with a molecular weight of 527 Daltons. Argatroban was FDA-cleared in 1997 for thrombosis prophylaxis and treatment and for anticoagulation during cardiac catheterization for patients with HIT.^{59,60}

For patients with HIT, the physician initiates the argatroban intravenous infusion at 2 $\mu\text{g}/\text{kg}/\text{min}$ or in patients with hepatic

disease at 0.5 $\mu\text{g}/\text{kg}/\text{min}$. During percutaneous cardiac intervention, a bolus of 350 $\mu\text{g}/\text{kg}$ is given over 3 to 5 minutes, followed by an infusion at 25 $\mu\text{g}/\text{kg}/\text{min}$. Argatroban is cleared by the liver and excreted in stool. There is a 5% general bleeding risk and no direct reversal agent; however, the half-life is 50 minutes, and argatroban clears completely in 2 to 4 hours.

Bivalirudin, a Recombinant Analogue of Leech Saliva Hirudin

Bivalirudin (Angiomax; The Medicines Company, Parsippany, NJ) is a synthetic 20-amino acid (2180 Daltons molecular weight) peptide derivative of the active site of hirudin, an anticoagulant produced in trace amounts by the medicinal leech *Hirudo medicinalis*. Bivalirudin was cleared by the FDA in 2000 for use as an anticoagulant in patients with unstable angina at risk for HIT who are undergoing percutaneous coronary intervention.⁶¹

Bivalirudin is intended for use with concurrent aspirin therapy at a dosage of 325 mg/day and has been studied only in patients receiving aspirin.⁶² Physicians provide an intravenous bolus dose of 0.75 mg/kg bivalirudin, followed by an infusion of 1.75 mg/kg/hr for the duration of the percutaneous cardiac intervention. After 4 hours, an additional intravenous infusion may be given at a rate of 0.2 mg/kg/hr for 20 hours.

The rate of major hemorrhage with bivalirudin is 4%. There is no reversal agent; however, in patients with normal renal function, the half-life is 25 minutes. The dosage is decreased in patients with reduced creatinine clearance or elevated serum creatinine.^{63,64}

Dabigatran, an Oral Direct Thrombin Inhibitor

Oral dabigatran etexilate (Pradaxa; Boehringer Ingelheim, Ingelheim, Germany) is a prodrug that converts upon ingestion to active dabigatran, a reversible DTI that binds both free and clot-bound thrombin. Dabigatran's efficacy and safety appear to match those of LMWH and Coumadin, and it has no known interaction with food. It is cleared by the kidneys, has a half-life

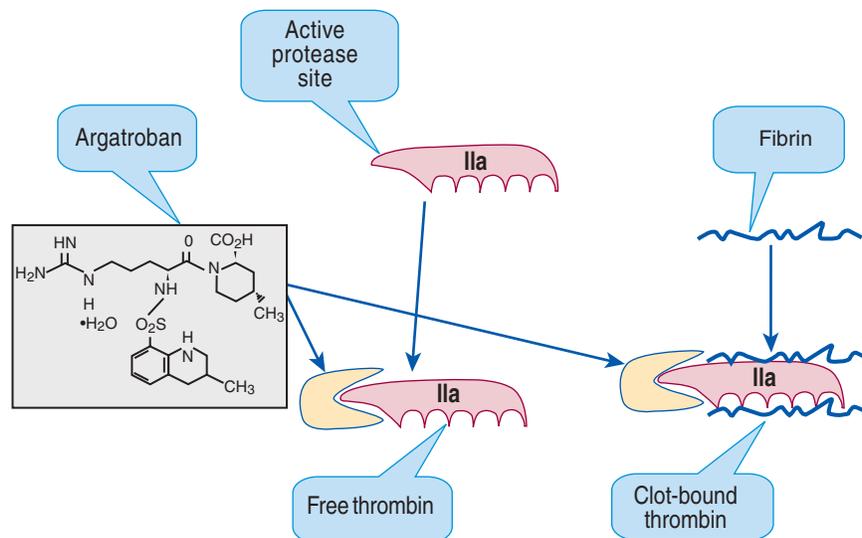


Figure 43-8 Argatroban inhibits the active site of free and clot-bound thrombin. Antithrombin is not involved in this reaction. Bivalirudin and dabigatran inhibit thrombin by interacting at the same site but with their own specific binding characteristics.

of 12 to 17 hours, and is not metabolized by liver cytochrome enzymes; however, it does affect the P-glycoprotein transport system, which can impact drug-drug interactions.⁶⁵ Dabigatran was cleared by the European Medicines Agency and Canada Health in the spring of 2008 for VTE prophylaxis following total knee or total hip replacement surgery at dosages of 220 mg/day, or 150 mg/day in the elderly or those with moderate renal impairment. In October 2010, the U.S. FDA cleared dabigatran for prevention of ischemic stroke in nonvalvular atrial fibrillation. In renal disease the half-life may be prolonged to as much as 60 hours, and in overdose-caused hemorrhage there is no known reversal agent.⁶⁶⁻⁷⁰

Measuring Direct Thrombin Inhibitor Therapy

Argatroban and bivalirudin prolong the thrombin time, PT, PTT, and ACT.⁷¹ For nonsurgical therapy, distributors recommend assaying with the PTT using the target therapeutic range of 1.5 to 3 times the mean of the laboratory reference interval (but not more than 90 seconds). Blood is collected 2 hours after the initiation of intravenous therapy for argatroban and 4 hours after therapy initiation for bivalirudin, and the dosage is adjusted to achieve a PTT within the therapeutic range. The ACT may be employed during cardiac catheterization or cardiac surgery. During these procedures, the target ACT for bivalirudin therapy is 320 to 400 seconds (median normal value, 98 seconds).

In instances in which the baseline PTT is prolonged by lupus anticoagulant, factor inhibitors, or factor deficiencies, the ecarin clotting time (ECT) is a potential alternative for assaying argatroban and bivalirudin. Ecarin (Ecarinase; Pentapharm, Basel, Switzerland) is an enzyme extracted from *Echis carinatus* venom that converts prothrombin to intermediate *meizothrombin*, which converts fibrinogen to fibrin. Argatroban and bivalirudin bind meizothrombin and generate a linear, dose-dependent prolongation of the ECT. Aside from DTIs, the ECT is prolonged only by abnormally low prothrombin or fibrinogen activity.

The oral direct thrombin inhibitor dabigatran is prescribed without routine monitoring. The clinical situations listed in [Box 43-1](#), however, may necessitate measuring dabigatran drug levels. Dabigatran prolongs the thrombin time, PTT, and ECT.⁷² The standard thrombin time is exceptionally sensitive to dabigatran and is convenient for ruling out dabigatran, because a normal thrombin time indicates that no dabigatran is present. A prolonged thrombin time may indicate that dabigatran is present, but it does not indicate the plasma concentration. The PTT generates a “curvilinear” response to dabigatran and is unreliable at low levels; additionally, there is considerable variability in sensitivity to dabigatran among PTT reagents.⁷³

The ECT and the ecarin chromogenic assay (ECA; Stago, Asnières sur Seine, France) provide a reliable, linear response to dabigatran, except at low concentrations.⁷⁴ Also, a modification of the thrombin time called the plasma-diluted thrombin time (Hemoclot Direct Thrombin Inhibitor Assay; Aniar Hyphen, West Chester, OH) and a chromogenic assay that is based on the thrombin time (Biophen DTI [chromogenic] Assay; Aniar Hyphen, West Chester, OH) are available.⁷⁵ ECT,

ECA, plasma-diluted thrombin time, and Biophen DTI, all of which require a sodium citrate plasma sample, await FDA clearance and are currently restricted to *research use only*.⁷⁶⁻⁷⁸

MEASURING ANTIPLATELET THERAPY USING PLATELET ACTIVITY ASSAYS

Intravenous Glycoprotein IIb/IIIa Inhibitors Are Used During Cardiac Catheterization

Glycoprotein IIb (α_{IIb}) and glycoprotein IIIa (β_3) are present on the membrane of resting platelets. Upon activation by any agonist, these molecules join to form glycoprotein IIb/IIIa ($\alpha_{IIb}\beta_3$) heterodimers, receptors that bind fibrinogen and von Willebrand factor through their arginine-glycine-aspartic acid (RGD) sequences. Fibrinogen binding to $\alpha_{IIb}\beta_3$ supports the key step of in vivo platelet aggregation (Chapter 13). The intravenous glycoprotein IIb/IIIa inhibitors (GPIs) abciximab, eptifibatid, and tirofiban fill $\alpha_{IIb}\beta_3$ receptor sites and block fibrinogen or von Willebrand factor binding, thereby preventing platelet aggregation⁷⁹ ([Figure 43-9](#)). Cardiologists use intravenous GPIs to maintain vascular patency during cardiac catheterization and intracoronary stent placement.⁸⁰

Before GPI infusion, the PT, PTT, ACT, hemoglobin, hematocrit, and platelet count are determined to detect any hemostatic or hematologic abnormality.⁸¹ During infusion, the PTT and ACT are maintained within the UFH therapeutic range as determined by the laboratory. Platelet counts are performed at 2 hours, 4 hours, and 24 hours following the initial bolus. If the platelet count drops by 25% or more, UFH and GPI are discontinued and the platelet count is monitored daily until it returns to within the reference interval. GPI efficacy may be measured using the Multiplate analyzer (DiaPharma, West Chester, OH; Roche Diagnostics Corporation, Indianapolis, IN) or VerifyNow IIb/IIIa assay (Accumetrics, San Diego, CA; International Technidyne Corporation, Edison, NJ).

Abciximab (ReoPro; Eli Lilly and Company, Indianapolis, IN) is the 47,615 Dalton Fab fragment of a mouse monoclonal antibody specific for $\alpha_{IIb}\beta_3$ that effectively fills the receptor site.⁸² The dose is 0.25 mg/kg given by intravenous bolus administered 10 to 60 minutes before the start of cardiac catheterization, followed by continuous infusion of 0.125 $\mu\text{g}/\text{kg}/\text{min}$ for up to 12 hours. Abciximab is always coadministered with UFH and aspirin.

Eptifibatid (Integrilin; Schering Corporation, Kenilworth, NJ) is an 832 Dalton heptapeptide GPI. It is coadministered with aspirin and UFH. An intravenous bolus of 180 $\mu\text{g}/\text{kg}$ is given as soon as possible after initial diagnosis, and a continuous intravenous drip of 2 $\mu\text{g}/\text{kg}/\text{min}$ is continued for up to 96 hours following the initial bolus, including throughout cardiac catheterization.

Tirofiban hydrochloride (Aggrastat; Baxter Healthcare Corporation, Deerfield, IL) is a 495 Dalton non-protein GPI that is coadministered with aspirin and UFH. It is administered intravenously at an initial rate of 0.4 $\mu\text{g}/\text{kg}/\text{min}$ for 30 minutes and then continued at 0.1 $\mu\text{g}/\text{kg}/\text{min}$ throughout cardiac catheterization and for 12 to 24 hours after catheterization. Tirofiban is excreted through the kidney, so the dosage is halved when the creatinine clearance is less than 30 mL/min.

Aspirin, Clopidogrel, Prasugrel, and Ticagrelor Reduce the Incidence of Arterial Thrombosis

The most commonly prescribed oral antiplatelet drugs are aspirin, clopidogrel (Plavix; Sanofi-Aventis, Bridgewater, NJ; Bristol-Myers Squibb, New York, NY), prasugrel (Effient; Eli Lilly and Company, Indianapolis, IN), and ticagrelor (Brilinta; AstraZeneca, Wilmington, DE). Aspirin irreversibly acetylates the platelet enzyme cyclooxygenase at the serine in position 529 (Figure 43-9). The serine-bound acetyl group sterically hinders the access of arachidonic acid to its reactive site within the cyclooxygenase molecule. This prevents production of platelet-activating thromboxane A_2 through the eicosanoid synthesis pathway (Chapter 13).⁸³ Acetylation is irreversible; the eicosanoid synthesis pathway is shut down for the remainder of the life of the platelet.

In contrast, clopidogrel, prasugrel, and ticagrelor are generally considered to be members of the thienopyridine drug family, though ticagrelor is actually a purine analogue. Thienopyridines occupy the platelet membrane adenosine diphosphate (ADP) receptor $P2Y_{12}$, suppressing the normal platelet aggregation and secretion response to the activating ligand (agonist) ADP. Clopidogrel and prasugrel are irreversible inhibitors, whereas ticagrelor is a reversible inhibitor.

Aspirin is often prescribed alone at 81 or 325 mg/day to prevent myocardial infarction and ischemic cerebrovascular disease in patients with stable or unstable angina,^{84,85} AMI,⁸⁶ transient cerebral ischemia,⁸⁷ peripheral vascular disease,⁸⁸ or stroke.^{89,90} In healthy people, aspirin prophylaxis annually prevents four thrombotic events per 1000 individuals treated, although it carries a risk of bleeding.⁹¹⁻⁹³

Clopidogrel is prescribed at 75 mg/day together with aspirin at 81 or 325 mg/day. Clopidogrel is a prodrug, and patients

appear to have varying responses to the fixed dose of clopidogrel, which raises the need for routine laboratory measuring using platelet function assays. Patients who possess a genetic variant of the CYP2C19 liver enzyme that activates clopidogrel may not get the full therapeutic effect. The variant polymorphism may be identified via molecular diagnostic techniques or phenotypically, as described in the next section.

Prasugrel was cleared by the FDA in July 2009.⁹⁴ It is administered as an oral prodrug that is converted in the liver via several cytochrome P-450 pathways to an active metabolite whose elimination half-life is about 7 hours. Treatment begins with a single 60-mg oral loading dose and continues at 10 mg daily, or 5 mg daily for patients who weigh less than 60 kg. Prasugrel is to be taken with aspirin at 81 mg or 325 mg daily and appears to require no laboratory measuring. However, up to 14% of patients may not achieve the full effect of prasugrel due to a genetic variant of the CYP2C19 liver enzyme needed to activate the drug. Drug interactions that increase or decrease the activity of prasugrel are important to identify. Prasugrel carries a higher risk of bleeding than clopidogrel and may be associated with an increased risk of solid tumors.

Ticagrelor was cleared in July 2011 to be coadministered with aspirin. Ticagrelor is a prodrug whose main active metabolite is formed rapidly via the CYP3A4 liver enzyme. It is provided in 90-mg tablets. Therapy is begun with two tablets totaling 180 mg, taken with one 325-mg aspirin tablet, followed by 90 mg of ticagrelor twice a day and one aspirin a day, not to exceed 100 mg. Ticagrelor reaches full effectiveness in 1.5 hours and maintains steady state for at least 8 hours. Drug interactions that increase or decrease the activity of ticagrelor are important to identify.

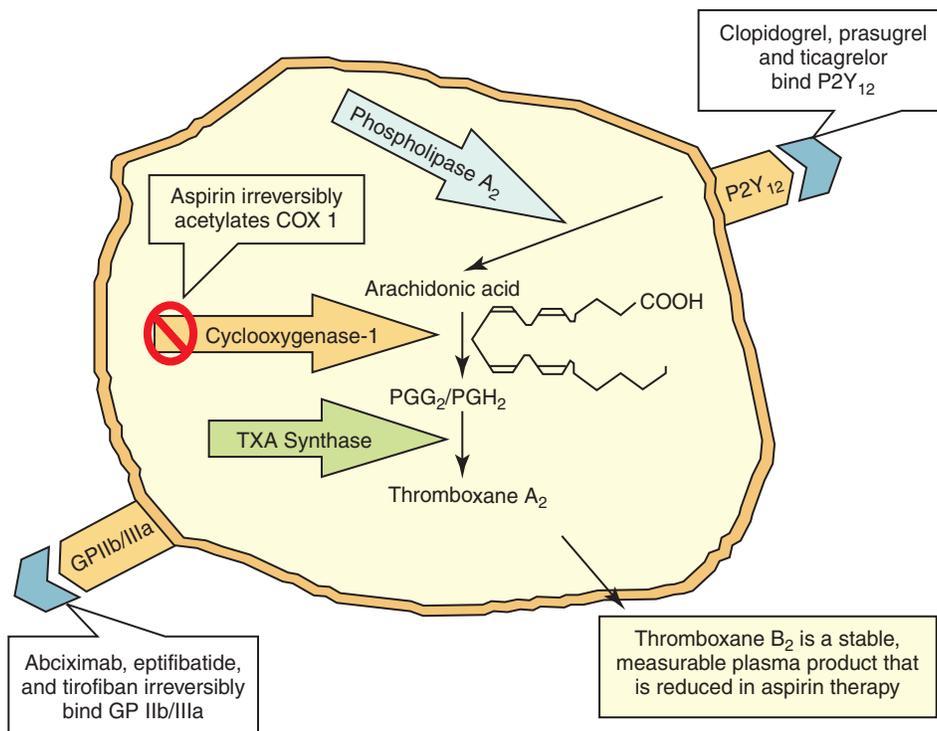


Figure 43-9 Antiplatelet drugs employ three mechanisms to inactivate platelets. Aspirin irreversibly acetylates and inactivates cyclooxygenase 1 (COX-1). Clopidogrel (irreversible), prasugrel (irreversible), and ticagrelor (reversible) bind the adenosine diphosphate (ADP) receptor, $P2Y_{12}$. Intravenous abciximab, eptifibatide, and tirofiban bind the fibrinogen binding site, glycoprotein (GP) IIb/IIIa. PGG_2 , Prostaglandin G_2 ; PGH_2 , prostaglandin H_2 ; TXA synthase, thromboxane A_2 synthase.

Variable Aspirin and Clopidogrel Response and Laboratory Measuring of Antiplatelet Resistance

Several investigations confirm that 10% to 20% of people who are taking aspirin generate an inadequate response as measured in the laboratory by light transmittance platelet aggregometry or whole-blood impedance aggregometry using arachidonic acid as the agonist. Inadequate response to aspirin has been termed *aspirin resistance*.⁹⁵⁻⁹⁸ Likewise, the response to clopidogrel as measured by aggregometry using ADP as the agonist varies markedly among patients, and the results of this assay may be used to adjust clopidogrel dosage.⁹⁹ Mechanisms that explain aspirin resistance and clopidogrel response variation are currently under study. Unlike treatment with aspirin and clopidogrel, prasugrel and ticagrelor therapy may show less interpatient variation. Aggregometry is the reference method for determining aspirin and clopidogrel responses, but several more rapid assays are available.

- **VerifyNow:** The Accumetrics *VerifyNow* (Accumetrics, San Diego, CA; International Technidyne Corporation, Edison, NJ) system is designed for point-of-care testing and uses light transmittance aggregometry to individually test for platelet aggregation responses to aspirin, clopidogrel, prasugrel, ticagrelor, and GPIs.¹⁰⁰ For each assay a cartridge is provided that contains the desired agonist and fibrinogen-coated beads. *VerifyNow* Aspirin uses arachidonic acid as its agonist. *VerifyNow* P2Y₁₂ uses ADP. *VerifyNow* IIb/IIIa uses thrombin receptor-activating polypeptide (TRAP), which activates platelets by binding to the thrombin receptor *protease-activated receptor 1* (PAR1; Chapter 13). The laboratory establishes reference interval limits and therapeutic target limits for each assay. Results that are outside the therapeutic target range indicate possible treatment failure and the need to revise dosage or change to a new antiplatelet drug. All three Accumetrics *VerifyNow* systems are FDA cleared.
- **Multiplate:** The Multiplate (DiaPharma, West Chester, OH; Roche Diagnostics Corporation, Indianapolis, IN) analyzer is automated for point-of-care testing and uses impedance aggregometry to simultaneously or individually test for platelet aggregation responses to aspirin, thienopyridines, and GPIs. The Multiplate requires 300 μ L of whole blood. The aspirin resistance assay uses arachidonic acid as its agonist, the thienopyridine response assay uses ADP, and the GPI response uses TRAP. The instrument integrates three aggregometry parameters—*aggregation velocity*, *maximum aggregation*, and *area under the aggregation curve*—to produce measurement units. The local laboratory establishes reference interval limits and expected therapeutic target ranges. Results that are outside the therapeutic target range indicate possible treatment failure and the need to revise dosage or change to a new antiplatelet drug. The Multiplate is available in Europe and was cleared by the FDA for use in the United States in August 2012.
- **Plateletworks:** The Plateletworks assay from Helena Laboratories (Beaumont, TX) determines the percent platelet aggregation in whole blood. Whole blood is added to EDTA tubes

coated with the agonists ADP or collagen, plus plain EDTA tubes. The practitioner performs platelet counts on the plain tube (baseline) and the agonist-treated EDTA tubes using an impedance-based electronic cell counter. The differences, expressed as percentages, indicate the degree of platelet aggregation triggered by each agonist. An expected effect of antiplatelet drug therapy would be a platelet aggregation response that is reduced 40% to 60% from a normal response.

- **PFA-100** (Siemens Medical Solutions USA, Inc., Malvern, PA). The PFA-100 system uses two cartridges. The first provides an aperture impregnated with collagen and epinephrine, and the second provides an aperture impregnated with collagen and ADP. The operator pipettes 800 μ L whole blood per cartridge and places the cartridge on the instrument.¹⁰¹ The specimen passes through the aperture until activation by the agonist causes occlusion of the aperture, generating a parameter called *closure time*. The PFA-100 tests only for aspirin resistance. A closure time that is shorter than the anticipated therapeutic range for aspirin indicates resistance.
- **AspirinWorks** (Corgenix Medical Corporation, Broomfield, CO): The AspirinWorks immunoassay measures a urine metabolite of platelet eicosanoid synthesis and thromboxane A₂ activation (Chapter 13). Hepatocyte *11-hydroxy-thromboxane dehydrogenase* acts upon stable platelet-derived plasma thromboxane B₂, the end product of eicosanoid synthesis and the stable analogue of thromboxane A₂, to produce water-soluble 11-dehydrothromboxane B₂.⁹⁹ The urine concentration of 11-dehydrothromboxane B₂ is sufficient for measurement without extraction and, because platelets seem to be its primary source, proportionally reflects platelet activity within the previous 12 hours. Urine levels of 11-dehydrothromboxane B₂ frequently are elevated in atherosclerosis; after stroke, transient ischemic attack, or intracerebral hemorrhage; and in atrial fibrillation. Levels of 11-dehydrothromboxane B₂ typically are decreased in patients receiving aspirin therapy, even in those with atherosclerosis, myocardial infarction, and atrial fibrillation, but appear to remain normal in patients who have aspirin resistance.

FUTURE OF ANTITHROMBOTIC THERAPY

Antithrombotic therapy, unchanged for more than 50 years, is likely to see further changes between 2014 and 2020. Several oral anticoagulants currently under development or awaiting clearance are likely to replace Coumadin, the heparins, and fondaparinux. Likewise, a series of new and emerging antiplatelet drugs will augment the time-honored aspirin tablet. The work of the clinical laboratory will reflect these changes, moving from the PT and PTT to chromogenic anti-factor Xa, modifications of the thrombin time, the ecarin clotting time, chromogenic assays, and new molecular assays. Antiplatelet response measuring will grow in convenience and take advantage of flow cytometry, immunoassays, and molecular assays that are currently in development.

SUMMARY

- Coumadin was developed in 1940 by Link and was first used in 1952. It prevents VTE, but it has a narrow therapeutic range, and an overdose causes hemorrhage. Coumadin therapy is monitored by the PT assay and reported as an INR. PT measurement is available on portable point-of-care instrumentation. Anticoagulation clinics are available to facilitate Coumadin monitoring and provide patient education and support.
- UFH is administered intravenously to provide immediate control of coagulation. Its therapeutic effect is monitored using the PTT, which requires the laboratory practitioner to develop a therapeutic range in seconds keyed to the chromogenic anti-factor Xa heparin assay. PTT results are subject to several interferences. Heparin is also used during cardiac catheterization or cardiac surgery that requires extracorporeal circulation. In these acute settings it is monitored by the ACT for its point-of-care and sensitivity to high heparin dose capabilities.
- LMWH and fondaparinux substitute for UFH and are administered subcutaneously for both prophylaxis and therapy. Both provide near-complete bioavailability, predictable dose response, and longer half-lives than UFH. LMWH and fondaparinux therapy require laboratory measurement only in patients with renal insufficiency, pregnant women, obese patients, children, and underweight adults using the chromogenic anti-factor Xa heparin assay.
- Rivaroxaban, apixaban, and edoxaban are the first to come to market of several oral direct-acting anti-factor Xa anticoagulants that require little laboratory measurement. New measurement techniques include the chromogenic anti-factor Xa assays using rivaroxaban, apixaban, and edoxaban calibrators and controls.
- The intravenous DTIs argatroban and bivalirudin directly bind thrombin without involving antithrombin and are substituted for heparin in patients with HIT. Intravenous DTI therapy is monitored using the PTT or ECT, and all affect the PT results during switchover to Coumadin therapy. At higher doses, as used during interventional procedures, these drugs are monitored by the ACT.
- Dabigatran is an oral DTI that requires minimal laboratory measurement. Dabigatran may be measured using the PTT, ECT, ECA, and plasma-diluted thrombin time.
- The antiplatelet drugs aspirin, clopidogrel, prasugrel, and ticagrelor are used after arterial thrombotic events to prevent repeat AMI, stroke, and PAO. Patient responses to aspirin and clopidogrel therapy vary. Response variation is detected using platelet aggregometry, the Accumetrics VerifyNow system, the Multiplate system, Helena's Plateletworks, the PFA-100 or the AspirinWorks assay; the latter two measure aspirin only.
- The intravenous antiplatelet drugs abciximab, eptifibatide, and tirofiban are used during cardiac catheterization to maintain vascular patency. Because they may cause thrombocytopenia, the platelet count is monitored carefully. Their efficacy may be monitored using the Accumetrics VerifyNow system, Helena's Plateletworks, or the Multiplate system.

Now that you have completed this chapter, go back and read again the case study at the beginning and respond to the questions presented.

REVIEW QUESTIONS

Answers can be found in the Appendix.

1. What is the PT/INR therapeutic range for Coumadin therapy when a patient has a mechanical heart valve?
 - a. 1 to 2
 - b. 2 to 3
 - c. 2.5 to 3.5
 - d. Coumadin is not indicated for patients with mechanical heart valves
2. Monitoring of a patient taking Coumadin showed that her anticoagulation results remained stable over a period of about 7 months. The frequency of her visits to the laboratory began to decrease, so the period between testing averaged 6 weeks. This new testing interval is:
 - a. Acceptable for a patient with stable anticoagulation results after 6 months
 - b. Unnecessary, because monitoring for patients taking oral anticoagulants can be discontinued entirely after 4 months of stable test results
 - c. Too long even for a patient with previously stable test results; 4 weeks is the standard
 - d. Acceptable as long as the patient performs self-monitoring daily using an approved home testing instrument and reports unacceptable results promptly to her physician
3. What is the greatest advantage of point-of-care PT testing?
 - a. It permits self-dosing of Coumadin
 - b. It is inexpensive
 - c. It is convenient
 - d. It is precise
4. You collect a citrated whole-blood specimen to monitor UFH therapy. What is the longest it may stand before the plasma must be separated from the cells?
 - a. 1 hour
 - b. 4 hours
 - c. 24 hours
 - d. Indefinitely

5. What test is used to monitor high-dose UFH therapy in the cardiac catheterization lab?
 - a. PT
 - b. PTT
 - c. Bleeding time
 - d. ACT
6. What test is used most often to monitor UFH therapy in the central laboratory?
 - a. PT
 - b. PTT
 - c. ACT
 - d. Chromogenic anti-factor Xa heparin assay
7. What test is used most often to monitor LMWH therapy in the central laboratory?
 - a. PT
 - b. PTT
 - c. ACT
 - d. Chromogenic anti-factor Xa heparin assay
8. What is an advantage of LMWH therapy over UFH therapy?
 - a. It is cheaper
 - b. It causes no bleeding
 - c. It has a stable dose response
 - d. There is no risk of HIT
9. In what situation is an intravenous DTI used?
 - a. DVT
 - b. HIT
 - c. Any situation in which Coumadin could be used
 - d. Uncomplicated AMI
10. What laboratory test may be used to monitor intravenous DTI therapy when PTT results are unreliable?
 - a. PT
 - b. ECT
 - c. Reptilase clotting time
 - d. Chromogenic anti-factor Xa heparin assay
11. What is the reference method for detecting aspirin or clopidogrel resistance?
 - a. Platelet aggregometry
 - b. AspirinWorks
 - c. VerifyNow
 - d. PFA-100
12. What is the name of the measurable platelet activation metabolite used in the AspirinWorks assay to monitor aspirin resistance?
 - a. 11-dehydrothromboxane B₂
 - b. Arachidonic acid
 - c. Thromboxane A₂
 - d. Cyclooxygenase
13. Which of the following is an intravenous antiplatelet drug used in the cardiac catheterization laboratory?
 - a. Abciximab
 - b. Ticagrelor
 - c. Prasugrel
 - d. Clopidogrel
14. Which of the following is a newly developed oral anticoagulant?
 - a. Argatroban
 - b. Lepirudin
 - c. Bivalirudin
 - d. Rivaroxaban
15. Which of the following is *not* a point-of-care instrument for the measurement of PT?
 - a. CoaguChek XS PT
 - b. Gem PCL Plus
 - c. Cascade POC
 - d. Multiplate

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44

Hemostasis and Coagulation Instrumentation

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OUTLINE

Historical Perspective Assay End-Point Detection Principles

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Photo-Optical End-Point Detection

Nephelometric End-Point Detection

Chromogenic End-Point Detection

Immunologic Light Absorbance End-Point Detection

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Improved Accuracy and Precision

Random Access Testing

Improved Reagent Handling

Improved Specimen Management

Expanded Computer Capabilities

Other Automated Features

Specimen Quality Set Points

Instrument Malfunction

Flags

Advantages and Disadvantages of Detection Methods

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Platelet Function Analyzers

Molecular Coagulation Testing

Selection of Coagulation Instrumentation

Currently Available Instru-

OBJECTIVES

After completion of this chapter, the reader will be able to:

1. Describe testing methodologies previously considered as specialized that are now routinely available on coagulation analyzers.
2. Identify testing applications for various coagulation analyzers.
3. Explain the methods of clot detection used by each type of coagulation analyzer presented.
4. List common instrument flags that alert operators to specimen and instrument problems.
5. Describe the advantages and disadvantages of each method of clot detection.
6. Distinguish the characteristics of manual, semiautomated, and automated coagulation analyzers.
7. Identify key performance characteristics that should be evaluated when selecting the most appropriate coagulation analyzer for an individual laboratory setting.
8. Explain the purpose of incorporating platelet function testing analyzers into the routine coagulation laboratory.
9. Identify the role of platelet aggregation in the coagulation laboratory.
10. Describe the methods available for molecular testing in the clinical lab and the analytes that can be measured using these techniques.
11. Develop a model plan of action for objective evaluation of coagulation analyzers for purchase.
12. Explain the main purpose of point-of-care coagulation testing.

CASE STUDY

After studying the material in this chapter, the reader should be able to respond to the following case study:

A 35-year-old white man was admitted to the hospital with abdominal pain and tenderness, malaise, and a low-grade fever. A tentative diagnosis of cholecystitis was made, with possible surgical intervention considered. Pertinent medical history included tonsillectomy at age 6 and appendectomy at age 18 with no abnormal bleeding symptoms noted. The patient reported that he was taking no medications at this time. In anticipation of surgery, routine coagulation studies were ordered. When the specimen arrived in the laboratory, it was centrifuged, and it was noted that the plasma had a whitish, milky appearance. The specimen was processed by an automated analyzer using photo-optical end-point detection methodology, and the following results were obtained:

Test	Results	Reference Interval	Flags
Prothrombin time	16.7 sec	10.9–13.0 sec	Lipemia
Partial thromboplastin time	>150 sec	30.6–35.0 sec	Lipemia
Fibrinogen	245 mg/dL	190–410 mg/dL	Lipemia

Because the laboratory's policy is to retest after all abnormal coagulation results, the prothrombin time and partial thromboplastin time assays were repeated, and similar values were obtained.

1. Should the operator report the test results as shown?
2. What action should the operator take to address the lipemia flagging of this specimen?
3. Would you expect this patient to be at risk for bleeding based on these test results?

*The authors acknowledge the substantial contributions of David L. McGlasson, MS, MLS(ASCP), to Chapter 47 of the fourth edition of this textbook, many of which continue in this edition.

The coagulation laboratory is an ever-changing environment populated by automated analyzers that offer advances in both volume and variety of tests.¹ Hardware and software innovations provide for random access testing with multitest profiles. In the past, the routine coagulation test menu consisted of prothrombin time (PT) with the international normalized ratio (INR), partial thromboplastin time (PTT; also referred to as the activated partial thromboplastin time [APTT]), fibrinogen, thrombin time, and D-dimer assays. More specialized testing was performed in tertiary care institutions or reference laboratories employing medical laboratory scientists with specialized training. With the introduction of new instrumentation and test methodologies, coagulation testing capabilities have expanded significantly, so many formerly “specialized” tests can be performed easily by general medical laboratory staff. New instrumentation has made coagulation testing more standardized, consistent, and cost effective. Automation has not advanced, however, to the point of making coagulation testing foolproof or an exact science. Operators must develop expertise in correlating critical test results with the patient’s diagnosis and when monitoring antithrombotic therapy. Good method validation of procedures, cognitive ability, and theoretical understanding of the hemostatic mechanisms are still required to ensure the accuracy and validity of test results so that the physician can make an informed decision about patient care.

HISTORICAL PERSPECTIVE

Visual clot-based testing began in the eighteenth century. The first observation of blood clotting was from blood taken from the vein of a dog that was completely “jellied” in about 7 minutes. In 1780, Hewson measured that human blood clotted in 7 minutes using a basin to collect the blood. With the discovery of the microscope, scientists were able to observe visible clot formation and turbidity.

Many advances took place from 1822 to 1921. These included temperature control during clot formation, passing objects such as a fine needle through the blood to detect resistance, and using different sizes and shapes of glass tubes to view clot formation. In the early 1900s, researchers monitored the length of time it took whole blood to clot in a glass tube while it was being tilted, a precursor to the Lee-White clotting time (1913). These early clotting time tests depended on observing the clot directly (visually) or microscopically.

In 1910, the first clot detection instrument, the “Koaguloviskosimeter,” was developed by Kottman. This apparatus measured the change in viscosity of blood as it clotted. This process generated a voltage change that was recorded by a direct readout system. Voltage changes were plotted against time to measure clot formation.

Except for point-of-care testing and whole-blood platelet aggregometry, citrated plasma (usually platelet-poor plasma, plasma with a platelet count of less than 10,000/ μ L) has now replaced whole blood in coagulation instruments. However, the principle of interval to clot formation lives on.^{1,2}

Plasma coagulation testing began in 1920 when Gram added calcium chloride to anticoagulated plasma at 37° C. He measured the increasing viscosity of the blood during fibrin monomer polymerization, a principle used today in thromboelastography (TEG) and sonar clot detection, laying the groundwork for the PT and PTT (Chapter 42).³

In these early days and for many years hence, coagulation testing was typically performed by adding plasma and reagents to a glass tube held in a 37° C water bath. Clot formation was determined by visual inspection of the plasma as the tube was tilted, and a stopwatch was used to determine the time to clot formation. This is referred to as the “tilt-tube technique.”

Nephelometers, developed in 1920, were the first instruments for coagulation testing. These devices measure 90-degree light dispersion of a colloidal suspension. As plasma clots, a change in light scatter can be measured over time—a principle still in use today. Subsequent twentieth-century developments in clot detectors include manual loops, an electromechanical end-point using a movable electrode (BBL Fibrometer) or a rolling steel ball (Diagnostica Stago ST-4), and photo-optical clot detection (Coag-A-Mate, originally manufactured by General Diagnostics).

The 1950s saw the development of the BBL Fibrometer, an instrument that can still be found in coagulation laboratories, although it is no longer being manufactured. This instrument employed an electromechanical clot detection methodology that allowed laboratories to transition from the manual tilt tube or wire loop method to a more accurate semiautomated testing process.

Current coagulation instruments apply many of the clot detection principles of these early analytical systems. They either “observe” the clot formation (optical, nephelometric devices) or they detect the clot by “feel” (mechanical, viscosity-based devices). Although the detection principles remain the same, current instrumentation has been enhanced to eliminate variations in pipetting and end-point detection. They also allow multiple analyses to be performed simultaneously on a single specimen.⁴

The introduction of new coagulation methodologies as will be described below has further improved testing capabilities in the coagulation laboratory. Refinement of these methodologies has allowed the use of synthetic substrates and measurements of single proenzymes, enzymes, and monoclonal antibodies, which increases the ability to recognize the causes of disorders of hemostasis and thrombosis.⁵

ASSAY END-POINT DETECTION PRINCIPLES

The available coagulometers are automated or semiautomated. Semiautomated coagulometers require the operator to deliver test plasma and reagents manually to the reaction cuvette and limit testing to one or two specimens at a time. These are relatively inexpensive instruments, but their use requires considerable operator expertise.

Fully automated analyzers provide pipetting systems that automatically transfer reagents and test plasma to reaction vessels and measure the end-point without operator intervention (Table 44-1). Multiple specimens can be tested simultaneously.

TABLE 44-1 Levels of Coagulation Automation

Level	Description	Examples
Manual	All reagents and specimens are transferred manually by the operator. Temperature is maintained by a water bath or heat block; external measurement by operator may be required. End-point is determined visually by the operator. Timer is initiated and stopped by the operator.	Tilt tube Wire loop
Semiautomated	All reagents and specimens are transferred manually by the operator. Instrument usually contains a device for maintaining constant 37° C temperature. Analyzer may internally monitor temperature. Instrument has mechanism to initiate timing device automatically on addition of final reagent and mechanism for detecting clot formation and stopping the timer.	Fibrometer STart 4 Cascade M and M-4 BFT-II KC1 and KC4
Automated	All reagents are automatically pipetted by the instrument. Specimens may or may not be automatically pipetted. Analyzer contains monitoring devices and internal mechanism to maintain and monitor constant 37° C temperature throughout testing sequence. Timers are initiated and clot formation is detected automatically.	ACL TOP STA-R Evolution STA Compact and Compact CT Sysmex CA-530, CA-560, CA-620, CA-660, CA-1500, CA-7000 BCS XP CoaLAB

Automated coagulometers are expensive, and laboratory staff require specialized training to operate and maintain them. Regardless of technology, all semiautomated and automated analyzers offer better coagulation testing accuracy and precision than the manual methods.

Instrument methodologies used for coagulation testing are classified into five groups based on the end-point detection principle:

1. Mechanical
2. Photo-optical (turbidometric)
3. Nephelometric
4. Chromogenic (amidolytic)
5. Immunologic

Historically, clot detecting instruments were limited to a single type of end-point detection system such as the mechanical or photo-optical detection. Photo-optical detection in instruments that read at a fixed wavelength between 500 nm and 600 nm has become the most commonly used system in today's clinical instruments. With the advancements in coagulation testing, a second type of instrument was designed to read at 405 nm to perform the chromogenic (colorimetric) assays. With advancements in technology, changes made it possible to automate advanced procedures. However, laboratories were required to purchase multiple analyzers if they wanted to offer the wider range of clot-based and chromogenic testing methods. Since 1990, instrument manufacturers have successfully incorporated multiple detection methods into single analyzers, which allows a laboratory to purchase and train on only one instrument while still providing specialized testing capabilities.⁴ Immunologics have recently been incorporated into coagulation laboratories for specific analyte measurements.

Mechanical End-Point Detection

Electromechanical clot detection systems measure a change in conductivity between two metal electrodes in plasma. The BBL

Fibrometer was the first semiautomated instrument to be used routinely in the coagulation laboratory. The probe of this instrument has one stationary and one moving electrode. During clotting, the moving electrode enters and leaves the plasma at regular intervals. The current between the electrodes is broken as the moving electrode leaves the plasma. When a clot forms, the fibrin strand conducts current between the electrodes even when the moving electrode exits the solution. The current completes a circuit and stops the timer.⁶

Another mechanical clot detection method employs a magnetic sensor that monitors the movement of a steel ball within the test plasma. Two principles are used for the mechanical clot detection in the routinely used coagulation instruments. In one system, an electromagnetic field detects the oscillation of a steel ball within the plasma-reagent solution.³ As fibrin strands form, the viscosity starts to increase, slowing the movement (Figure 44-1). When the oscillation decreases to a predefined rate, the timer stops, indicating the clotting time of the plasma. This methodology is found on all Diagnostica Stago analyzers.

In the second system, a steel ball is positioned in an inclined well. The position of the ball is detected by a magnetic sensor. As the well rotates, the ball remains positioned on the incline. When fibrin forms, the ball is swept out of position. As it moves away from the sensor, there is a break in the circuit, which stops the timer. This technology can be found on AMAX and Destiny instruments distributed by Tcoag US, a division of Diagnostica Stago, as well as on the original Hemochron ACT instruments.

Mechanical methods are not affected by icteric or lipemic plasma. Mechanical methods also provide a sensitive end-point able to detect weak clots such as those formed in plasmas with low fibrinogen or a factor XIII deficiency where clots are not stabilized.

Photo-Optical End-Point Detection

Photo-optical (turbidometric) coagulometers detect a change in plasma optical density (OD, light transmittance) during

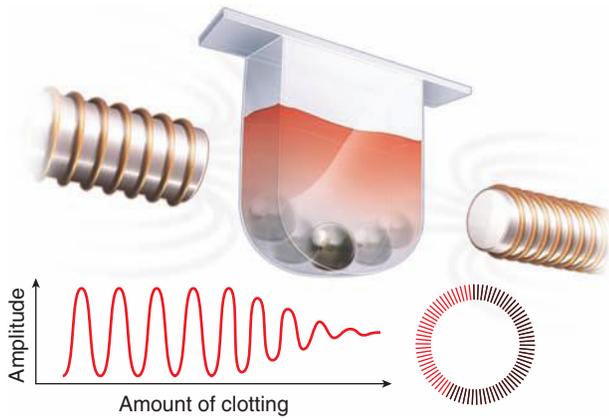


Figure 44-1 Viscosimetric (electromechanical) clot detection in a Diagnostica Stago analyzer. A steel ball oscillates in an arc from one side of the cuvette to the other. Movement is monitored continuously within a magnetic field. As the sample clots, viscosity rises and movement of the steel ball is impeded. Variation in amplitude stops the timer, and the interval is the clotting time.

clotting. Light of a specified wavelength passes through plasma, and its intensity (OD) is recorded by a photodetector. The OD depends on the color and clarity of the sample and is established as the baseline. Formation of fibrin strands causes light to scatter, allowing less light to fall on the photodetector, thus generating an increase in OD. When the OD rises to a predetermined variance from baseline, the timer stops indicating clot formation. Because the baseline OD is subtracted from the final OD, effects of lipemia and icterus are minimized. Many optical systems employ multiple wavelengths that discriminate and filter out the effects of icterus and lipemia. Most of the automated and semiautomated coagulation instruments developed since 1970 use photo-optical clot detection (Figure 44-2).

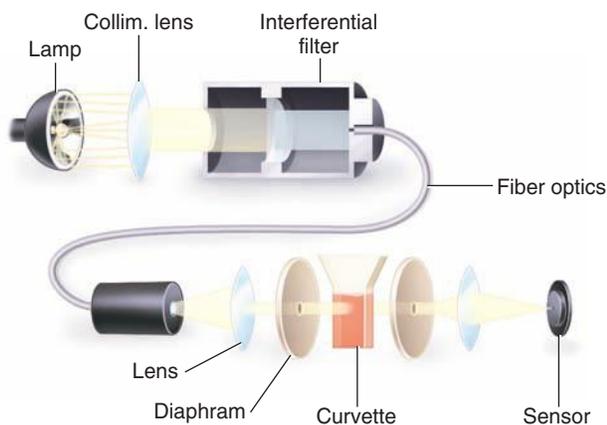


Figure 44-2 Photo-optical (turbidometric) clot detection. Polychromatic light is focused by a collimator and filtered to transmit a selected wavelength. Monochromatic light is transmitted by fiber optics and focused on the reaction cuvette. As fibrin forms, opacity increases and the intensity of light reaching the sensor decreases. *Collim.*, Collimator.

Nephelometric End-Point Detection

Nephelometry is a modification of photo-optical end-point detection in which 90-degree or forward-angle light scatter, rather than OD, is measured. A light-emitting diode produces incident light at approximately 600 nm, and a photodetector detects variations in light scatter at 90 degrees (side scatter) and 180 degrees (forward-angle scatter). As fibrin polymers form, side scatter and forward-angle scatter rise (Figure 44-3).^{4,7,8} The timer stops when scatter reaches a predetermined intensity, and the interval is recorded.

Nephelometry can be adapted to dynamic clot measurement. Continuous readings are taken throughout clotting, measuring the entire clotting sequence to completion and producing a clot curve or "signature."

Nephelometry was first applied to immunoassays. As antigen-antibody complexes (immune complexes) precipitate, the resulting turbidity scatters incident light.⁹ In reactions in which the immune complexes are known to be too small for detection, the antibodies are first attached to microlatex particles. In coagulation, coagulation factor immunoassays employ specific factor antibodies bound to latex particles. Nephelometry provides a quantitative, but not functional, assay of coagulation factors. Nephelometry is often employed in complex automated coagulometers because it allows for both clot-based assays and immunoassays. Nephelometry-style analyzers can be used to produce high-volume multiple-assay coagulation profiles.

Chromogenic End-Point Detection

Chromogenic (synthetic substrate, amidolytic) methodology employs a synthetic oligopeptide substrate conjugated to a chromophore, usually para-nitroaniline (pNA) (Chapter 42). Chromogenic analysis is a means for measuring specific coagulation factor activity because it exploits the factor's enzymatic (protease) properties. The oligopeptide is a series of amino acids whose sequence matches the natural substrate of the protease being measured.¹⁰⁻¹³ Protease cleaves the chromogenic substrate at the site binding the oligopeptide to the pNA, freeing the pNA. Free pNA is yellow; the OD of the solution is proportional to protease activity and is measured by a photodetector at 405 nm (Figure 44-4).

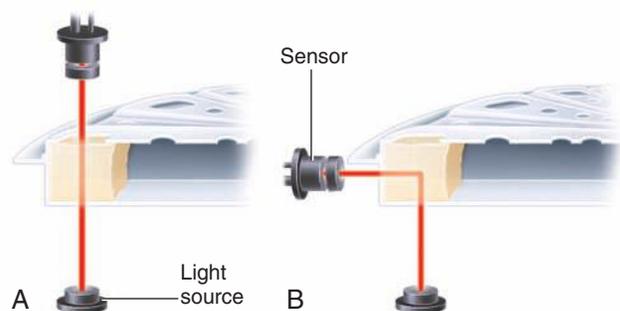


Figure 44-3 Nephelometric clot detection. **A**, Light from below passes through the sample in a cuvette to the detector above. **B**, As fibrin polymerizes, light is deflected and is detected at an angle from the original path.

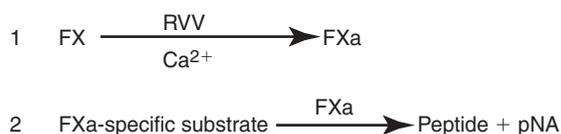


Figure 44-4 Method used in the DiaPharma chromogenic factor X assay as an example of a direct chromogenic assay. The method involves two stages. In stage 1, the activator Russell viper venom (RVV) activates factor X (FX) in the presence of calcium to factor Xa (FXa). In stage 2, the generated FXa hydrolyzes the chromogenic substrate S-2765, liberating the chromophore group para-nitroaniline (pNA). Free pNA is yellow in color. The color is then read photometrically at 405 nm. The amount of FXa generated, and thus the intensity of the color, is proportional to the FX activity in the sample over the assay range.

Assays based on a chromogenic end-point (rather than a clot-based assay) are useful to evaluate specimens from patients who have circulating inhibitors or who are on anticoagulant treatment because the inhibitors do not interfere in the chromogenic assay. For clot-based assays, the entire coagulation cascade is part of the test system. For chromogenic assays, the test is isolated to the specific chemical reaction in question.

The activity of coagulation factors and many other enzymes are measured by the chromogenic method directly or indirectly:

- *Direct chromogenic measurement:* OD is proportional to the activity of the substance being measured—for instance, protein C activity measured by a synthetic chromogenic substrate specific for protein C.
- *Indirect chromogenic measurement:* The protein or analyte being measured inhibits a target enzyme. It is the target enzyme that has activity directed toward the synthetic chromogenic substrate. The change in OD is inversely proportional to the concentration or activity of the substance being measured—for example, heparin in the anti-factor Xa assay.

Immunologic Light Absorbance End-Point Detection

Immunologic assays are the newest assays available in coagulation laboratories and are based on antigen-antibody reactions similar to those used in nephelometry as described previously. Latex microparticles are coated with antibodies directed against the selected analyte (antigen). Monochromatic light passes through the suspension of latex microparticles. When the wavelength is greater than the diameter of the particles, only a small amount of light is absorbed.¹⁴ When the coated latex microparticles come into contact with their antigen, however, the antigen attaches to the antibody and forms “bridges,” which causes the particles to agglutinate. As the diameter of the agglutinates increases relative to the wavelength of the monochromatic light beam, light is absorbed. The increase in light absorbance is proportional to the size of the agglutinates, which in turn is proportional to the antigen level.

Immunoassay technology became available on coagulometers in the 1990s and is used to measure a growing number of coagulation factors and proteins, such as D-dimer. These assays, which used to take hours or days to perform using traditional antigen-antibody detection methodologies

such as enzyme-linked immunosorbent assay or electrophoresis, now can be done in minutes on an automated analyzer.

ADVANCES IN COAGULATION TECHNOLOGY

Significant advances have been made in the capability and flexibility of coagulation instrumentation. Instruments previously required manual pipetting, recording, and calculating the results, which necessitated significant operator expertise, intervention, and time. Current technology allows a “walkaway” environment in which, after specimens and reagents are loaded and the testing sequence is initiated, the operator can move on to perform other tasks.

Clot detection methods have remained consistent, but with the advent of chromogenic- and immunologic-based assays other instrumentation needed to be incorporated into the coagulation laboratory. Multiple methodologies became incorporated into single analyzers to expand their test menu options. From instruments that performed only clot-based assays, clinical laboratory instruments were developed that could perform both clot-based and chromogenic-based assays on one platform.^{12,15-17} The next step was the development of a single instrument that could perform clotting and chromogenic and immunologic assays. Additional advances have included improved specimen and reagent storage and processing, increased throughput, and enhanced data management and result traceability.

Improved Accuracy and Precision

In the days of visual methods, coagulation assays were performed in duplicate to reduce the coefficient of variation, which generally exceeded 20%. Semiautomated instruments have improved upon precision, but the requirement for manual pipetting of plasma and reagents continues to necessitate duplicate testing. With the advent of fully automated instruments, precision has improved to the extent that single testing can be performed with confidence, halving material and reagent costs. Coefficients of variation of less than 5%, and even less than 1% for some tests, have been achieved. Initial accuracy and precision are established by in-lab method validation for all instrument and reagent combinations.

Random Access Testing

Automated coagulometers now provide random access testing. Through simple programming, a variety of tests can be run in any order on single or multiple specimens within a testing sequence. Previous automated analyzers were capable of running only one or two assays at a time, so batching was necessary. The disadvantage was that specimens with multiple orders had to be handled multiple times. For current automated analyzers, the ability to run multiple tests is limited only by the number of reagents that can be stored in the analyzer and the instrument’s ability to interweave tests requiring different end-point detection methodologies simultaneously, such as clot-based, chromogenic, and immunologic methods. Random access promotes profiling.

Improved Reagent Handling

Reduced Reagent and Specimen Volumes. Automated and semiautomated coagulometers now have the capability to perform tests on smaller sample volumes. Traditionally, PT assays required 0.1 mL of patient plasma and 0.2 mL of thromboplastin/calcium chloride reagent. PTT was measured using 0.1 mL of plasma, 0.1 mL of activated partial thromboplastin, and 0.1 mL of calcium chloride. Current analyzers can perform the same tests using one half or even one quarter the traditional volumes of reagents and patient specimens. This promotes the use of smaller specimen volumes, especially from pediatric patients or those from whom specimens are difficult to draw, and further reduces reagent costs.

Open Reagent Systems

A variety of reagents from numerous distributors are available for coagulation testing, and laboratory directors want the flexibility of selecting the reagents that best suit their needs without being restricted in their choices by the analyzers being used. Recognizing that the ability to select reagents independently of the test system is a high priority, instrument manufacturers have responded by developing systems that provide optimal performance with alternative manufacturer's reagents, provided that the reagents are compatible with the instrument's methodology.

Reagent Tracking

Many automated instruments keep records of reagent lot numbers and expiration dates, which makes it easier for the laboratory to maintain reagent integrity and comply with regulatory requirements. Additional features often include on-board monitoring of reagent volumes with flagging systems to alert the operator when an insufficient volume of reagent is present in relation to the number of specimens programmed to be run. Reagent bar coding supports record keeping because it tracks reagent properties and enables the operator to load coagulometers without stopping specimen analyses.

Improved Specimen Management

Primary Tube Sampling

Many coagulometers encourage the operator to place the primary collection tube on the instrument after centrifugation, which eliminates the need to separate the plasma into a secondary tube. In addition, instruments often accommodate multiple tube sizes. Significant time savings occur as a result of elimination of the extra specimen preparation step, and errors resulting from mislabeling of the aliquot tube are reduced.

Closed-Tube Sampling

Closed-tube sampling has improved the safety and efficiency of coagulation testing. After centrifugation, the tube is placed on the analyzer without removing the blue stopper. The cap is pierced by a needle that aspirates plasma without disturbing the red blood cell layer. Not only does closed-tube sampling save staff time, it also reduces the risk of specimen exposure through aerosols or spillage. Closure also promotes plasma pH stabilization. When closed-tube sampling is used, specimens

are visually checked for clots after centrifugation by looking for the presence of fibrin strands. For example, if the assay result is a short clotting time (or the corresponding coagulation tracing available on some instruments) is abnormal then the sample will be rimmed with wooden sticks to determine if a clot is present.

Flagging for Specimen Interferences

Some analyzers monitor the quality of the test specimen for interfering substances or unusual testing characteristics, such as hemolysis, lipemia, bilirubinemia (icterus), abnormal clotting patterns, or results that fall outside the linear range of the reference curve (values above the top point or below the bottom point of the calibration line). Flags warn the operator of potential errors so that problems can be resolved in a timely manner (see later).

Automatic Dilutions

Many instruments perform multiple dilutions on patient specimens, calibrators, or controls, eliminating the need for the operator to perform this task manually and reducing the potential for dilution errors. These conditions can be automatically programmed into the individual test setups on the analyzers being used.

Expanded Computer Capabilities

The computer circuitry of analyzers now incorporates internal data storage and retrieval systems. Hundreds of results can be stored, retrieved, and compiled into cumulative reports. Multiple calibration curves can be stored and accessed. Quality control files can be stored, which eliminates the time-consuming task of manually logging and graphing quality control values. Westgard rules can be applied, and failures are automatically flagged. Some analyzers feature automatic repeat testing when failures occur on the initial run. The quality control files can be reviewed or printed on a regular basis to meet regulatory requirements.

The programming flexibility of modern analyzers has enhanced the laboratory's opportunities to provide expanded test menus. Most advanced analyzers are preprogrammed with several routine test protocols ready for use. Specimen and reagent volumes, incubation times, and other testing parameters do not need to be predetermined by the operator but can be changed easily when necessary. Additional tests can be programmed into the analyzer by the user whenever needed, which allows for enhanced flexibility of the analyzer and reduces the need for laboratories to have multiple instruments.

Instrument interfacing to laboratory information systems and specimen bar coding capabilities have become a priority as facilities of all sizes endeavor to reduce dependence on manual record keeping. Bidirectional interfaces improve efficiency through the ability of the instrument to send specimen bar code information to the laboratory information systems and receive a response listing the tests that have been ordered. This eliminates the need for the operator to program each specimen and test.

Other Automated Features

A few additional features offered by current coagulation analyzers should be mentioned:

Improved flagging capabilities alert the operator when preset criteria have been exceeded (Box 44-1). Flags may indicate instrument malfunction such as cuvette jams, low reagent volume, and temperature errors, or a problem with the results such as values that exceed critical limits, inability to detect an accurate end-point, or values outside of the linear range.

Reflex testing is the automatic ordering of tests based on preset parameters or the results of prior tests. Instruments may make additional dilutions if the initial result is outside of the linearity limits, or supplementary tests can be run automatically if clinically indicated by the initial test result. The first result does not need to wait for review by the operator before follow-up action is taken.

Graphing of clot formation is provided on analyzers such as the ACL TOP (Instrumentation Laboratory). The graph is generated by an algorithm, a formula used to convert raw optical measurements into a clotting time. Besides determining the clotting time, it also smooths the raw data into a visible curve and uses the curve to check for clot integrity. Multiple checks are performed to ensure an accurate and reproducible result. Should the data not meet all of the acceptable criteria, an error flag is generated. The clot curve is examined to troubleshoot potential technical aberrations. The clot formation graph may also be used as a “signature” that correlates with the disease state. Figure 44-5 shows an example of a typical clot curve.

Specimen Quality Set Points

Specimen quality flags, such as the following, can be included on some coagulation instrumentation:

- *Clotted*: will cause falsely shortened clotting times because of premature activation of coagulation factors and platelets that generate FVIIa and thrombin.
- *Lipemia*: may cause falsely prolonged clotting times on OD instruments because of interference with light transmittance.
- *Hemolysis*: may cause falsely shortened clotting times because of premature activation of coagulation factors and platelets that generate FVIIa and thrombin.¹⁸

BOX 44-1 Warning Flags Available on Coagulometers

Instrument Malfunction Flags

Temperature error
Photo-optics error
Mechanical movement error
Probe not aspirating

Sample Quality Flags

Lipemia
Hemolysis
Icterus
Abnormal clot formation
No end-point detected

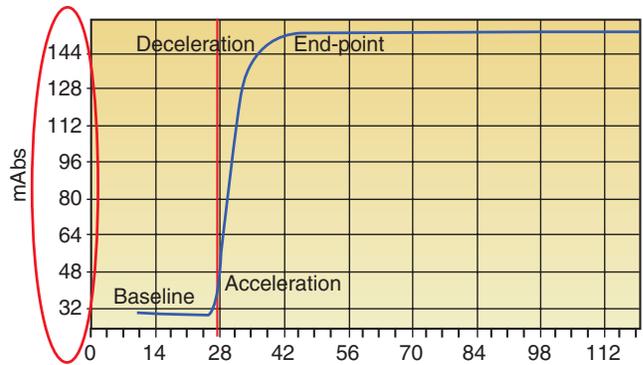


Figure 44-5 Clot signature produced by the ACL TOP analyzer (Instrumentation Laboratory). The clot curve consists of baseline, acceleration phase, deceleration phase, and end-point. The baseline is recorded before clotting occurs. Acceleration reflects clotting and is normally steep because clotting is rapid. The deceleration phase represents the decreasing rate of clot formation as all available fibrinogen converts to fibrin. The end-point is flat and stable, reflecting consumption of all fibrinogen. A key component in evaluating the clot curve is the y-axis, absorbance, which provides the clot interval when it reaches a defined minimum. Absorbance adjusts to compensate for baseline fibrinogen and interferences such as lipemia or icterus. (Image courtesy of Beckman-Coulter, Brea, CA.)

- *Icterus (bilirubinemia)*: indicates liver dysfunction that may lead to prolonged clotting times because of inadequate clotting factor production; also may interfere with OD instruments.
- *Abnormal clot formation*: may lead to falsely elevated clotting times because of instrument inability to detect an end-point.
- *No end-point detected*: indicates that the instrument was unable to detect clot formation; the specimen may need to be tested using an alternate methodology.

Instrument Malfunction Flags

Instrument malfunction flags, such as the following, can be included on some instrumentation:

- Temperature error
- Photo-optics error
- Mechanical movement error
- Probe not aspirating
- No end-point detected
- Specimen track error
- Cuvette jams

ADVANTAGES AND DISADVANTAGES OF DETECTION METHODS

Photo-optical end-point detection may be confounded by icterus or lipemia, which erroneously prolongs the clotting time because the change in OD is masked by the color or turbidity of the specimen. Some coagulation instruments may be unable to employ synthetic reagents because they are more translucent than reagents used to optimize the end-point detectors.¹⁹

All coagulation tests that use clot-based end-points need special considerations when interpreting results. There are many preanalytical variables that affect coagulation function, and the integrity of the entire coagulation cascade is relevant to

the final test result. Thus these assays are not highly specific. This would not be a function of chromogenic or immunologic end-point assays that do not rely on the cascade but rather are single analyte specific. Table 44-2 summarizes the advantages and disadvantages of each detection methodology.

POINT-OF-CARE TESTING

Point-of-care coagulation testing has been used since 1966 in the setting of cardiac surgery using the whole-blood activated clotting time (ACT) for heparin monitoring in the operating room (Chapter 43). Today point-of-care testing has expanded beyond the ACT. Most point-of-care instruments are handheld and permit near-patient testing, bedside testing, self-testing, and testing of infants. The instantaneous turnaround time of results, small sample volume, and portability of the instruments are conveniences appreciated by physicians and patients.

Anticoagulation clinics can be high-volume users of point-of-care testing. Patients on oral anticoagulant therapy with

vitamin K antagonists need to be monitored monthly (Chapter 43). Anticoagulation clinics provide this service in the outpatient setting using the prothrombin time/international normalized ratio (PT/INR) test on a point-of-care instrument. Quick test resulting allows for dose adjustments at the same clinic visit, eliminates patient waiting time, and allows time for patient education.

Other point-of-care coagulation tests include the PTT and thrombin clotting time with other assays such as fibrinogen likely available in the future.

Table 44-3 summarizes the variety of FDA-cleared point-of-care instruments and assays available.²⁰ Various end-point detection techniques are employed. Each instrument uses individual patented technology. The newer versions of these point-of-care instruments include touch screen, wireless transmission of results in real-time, and micro-blood volumes.

Point-of-care coagulation analyzers employ capillary (finger-stick) or anticoagulated whole blood (venipuncture). Typically a 10- to 50- μ L sample is transferred to a test cartridge and the cartridge is inserted into the test module. Other instruments

TABLE 44-2 Advantages and Disadvantages of Detection Systems

End-Point Detection Method	Advantages	Disadvantages
Mechanical	No interference from specimen lipemia or bilirubinemia (icterus) Ability to use specimen and reagent volumes as small as 25 μ L in some instruments Able to detect weak clots	Reliance on the integrity of the entire coagulation cascade Inability to observe graph of clot formation
Photo-optical	Good precision Increased test menu flexibility and specimen quality information when multiple wavelengths are used Ability to observe graph of clot formation with some instrumentation	Interference from lipemia, hemolysis, bilirubinemia, and increased plasma proteins; this issue has been addressed by some manufacturers with readings from multiple wavelengths May not detect short clotting times owing to long lag phase May not detect small friable clots that are translucent
Chromogenic	Ability to measure proteins that do not clot More specific than clot-based assays Expanded menu options to replace clottable assays affected by preanalytical variables such as heparin, thrombin inhibitors (e.g., argatroban, dabigatran) or FXa inhibitors (e.g., rivaroxaban) Most automated systems now have cost-effective chromogenic capabilities	Limited by wavelength capabilities of some instruments May need large test volume to be cost effective
Immunologic	Ability to automate tests previously available only with manual, time-consuming methods, such as enzyme-linked immunosorbent assays Expanded test menu capabilities	Limited number of automated tests available Higher cost of instruments and reagents May need to have additional instruments available to run routine tests in laboratories without automated coagulation analyzers that have random access capability
Nephelometric	Ability to measure antigen-antibody reactions for proteins present in small concentrations	Limited number of tests available Higher cost of reagents Need for special staff training

TABLE 44-3 A Comparison of Point-of-Care Instruments for Coagulation Testing

Manufacturer/ Instrument	Year	Testing	Specimen	Clot Detection	Includes QC
Abbott/ISTAT	2000	PT/INR, PTT, ACT	Whole Blood	Electrogenic	Yes
Alere/INRatio/INRatio2	2003/2008	PT/INR	Fingerstick	Electrochemical, impedance	Yes
Helena/Cascade POC	2000	PT/INR, PTT, ACT, low molecular weight heparin	Whole Blood/Fingerstick	Photo-mechanical	Yes
Helena/Actalyke Mini II	2004	ACT	Whole Blood	2-point electromechanical	Yes
Helena/Actalyke XL	2002	ACT	Whole Blood	2-point electromechanical	Yes
Instrumentation Lab/ Gem PCL Plus	2003	PT/INR, PTT, ACT	Whole Blood/Fingerstick	Mechanical end-point monitored optically	Yes
ITC/ProTime Micro Coagulation System	1995/2003/2006	PT/INR	Fingerstick	Mechanical clot	Yes
ITC/Hemochron Signature Elite	2005	PT/INR, PTT, ACT	Whole Blood/Fingerstick	Mechanical clot	Yes
ITC/Hemochron Signature +	2002	PT/INR, PTT, ACT	Whole Blood/Fingerstick	Mechanical clot	Yes
ITC/Hemochron Response	2000	PT/INR, PTT, ACT, TT	Whole Blood/Fingerstick	Mechanical clot	Yes
Medtronics/HMS Plus	1999	ACT, protamine titration	Whole Blood	Mechanical clot	Yes
Medtronics/ACT Plus	2003	ACT	Whole Blood	Mechanical clot	Yes
Roche/CoaguCheck XS PT Test System	2007	PT/INR	Whole Blood/Fingerstick	Amperometric	No
Roche/CoaguCheck XS Plus PT Test System	2007	PT/INR	Whole Blood/Fingerstick	Amperometric	Yes
Roche/CoaguCheck XS Pro PT Test System	2010	PT/INR	Whole Blood/Fingerstick	Amperometric	Yes
CoaguSense PT/INR Monitoring System	2010	PT/INR	Fingerstick	Mechanical	Yes

require nonanticoagulated whole blood, so higher volumes of blood are needed.

Before point-of-care instruments are placed in service, laboratory scientists validate the units against a reference method in a central lab using the plasma-based assays (Chapter 5). Because point-of-care assays and the plasma-based central laboratory assays can show a weak correlation, care must be taken to understand the differences between point-of-care and central lab results and to ensure that clinical decisions are consistent. In the case of the anticoagulation clinic, an INR that exceeds 4.0 or any unexpected INR change is confirmed with a venipuncture blood specimen tested by the plasma-based assay in the central lab.

WHOLE-BLOOD CLOTTING ASSAYS

The *TEG Thromboelastograph Hemostasis Analyzer System* (Haemoscope, Niles, IL, a division of Haemonetics) is an operator-dependent system that provides global hemostasis assessment. The TEG assesses both bleeding and thrombosis risk in patients. TEG analysis is useful in patients with hepatic disease, having liver transplant surgery, undergoing cardiac surgery, obstetrics patients, and trauma patients. Computerization

of TEG facilitates its usefulness in intraoperative hemostasis management, where it helps to predict the need for and to monitor clotting factor administration, platelet transfusion, fibrinolytic therapy, and antiplatelet therapy with medications such as aspirin or clopidogrel.

Citrated whole blood is pipetted into a cylindrical cup that oscillates by 4.75°. A stationary pin with a diameter 1 mm smaller than the cup's is suspended by a torsion wire in the cup. Kaolin (or another activator) is added to trigger clotting. As the blood clots, fibrin links the pin to the cup, and viscoelasticity changes are transmitted to the pin. The resulting pin torque generates an electrical signal from the torsion wire that is plotted as a function of time to produce a TEG tracing (Figure 44-6). The tracing is analyzed to determine the speed, strength, and stability of clot formation and the downstream effect of fibrinolysis. Viscoelasticity depends on procoagulant activity, cellular components (red blood cells, white blood cells, and platelets), and fibrinolysis and the interactions between these components. The trace furnishes real-time information about the evolving clot from platelet activation to initial fibrin formation, fibrin cross-linkage, and fibrinolysis.²¹ This is the only available whole blood assay that provides a comprehensive, global evaluation of functional hemostasis.

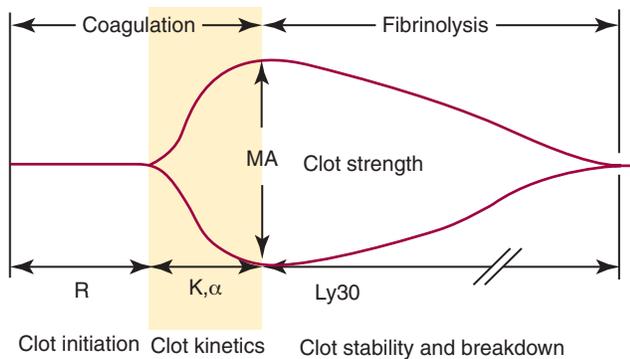


Figure 44-6 Thromboelastograph (TEG) tracing from the TEG 5000 Hemostasis Analyzer System. The TEG[®] analyzer produces results that document the interaction of platelets with proteins (enzymes, inhibitors, cofactors) of the coagulation pathway and the fibrinolytic system as a whole blood clot forms and eventually breaks down. The R value reflects the initial generation of thrombin and fibrin formation. The K value and angle reflect the rate of initial clot formation mediated by thrombin-activated platelets, fibrin generation, fibrin polymerization, and the developing strength and stabilization of the clot due to platelet function, fibrinogen level, and FXIII. The MA value is an indicator of fibrin-platelet interaction, and overall clot firmness and stability. LY is a kinetic measure of time to and extent of clot lysis. (This image is used with permission of the Haemoscope Corporation, a division of Haemonetics, Niles, IL.)

A newer version is the *ROTEM* (Tem Innovations GmbH, Munich, Germany). The enhancement of the *ROTEM* is that it is not sensitive to vibrations. In the *ROTEM*, a whole-blood sample is placed in a cuvette, and a suspended pin is immersed into the blood. One of various activators is added to trigger clotting. The pin rotates (the cup is stationary), and upon clot formation, the increased tension from fibrin binding the cup to the pin is detected by sensors. *ROTEM* parameters are the same as those described above for TEG.²² *ROTEM* reagents are available to assess factor and fibrinogen deficiency, platelet function, fibrinolysis, and anticoagulant influence on hemostasis.

PLATELET FUNCTION TESTING

The demand for rapid, cost-effective methods for the evaluation of platelet function has increased due to the need to monitor the efficacy of antiplatelet therapy such as aspirin, clopidogrel, and glycoprotein IIb/IIIa inhibitors used in cardiovascular patients. In addition, preoperative evaluation of platelet function is important in hemostatic management, particularly if the patient has a history of bleeding or if the patient is on antiplatelet medication.²³ Platelet function testing has been a challenge for the clinical laboratory because of the lack of reliable, accurate, and easy-to-perform methods. In addition, specimen procurement for platelet function testing plays an important role in the reliability and accuracy of the test. Platelet function historically has been assessed by the bleeding time test and platelet aggregation assays (Chapter 42). The bleeding time is technically demanding and is highly dependent on the technician performing the test. In addition, it fails

to correlate with intraoperative bleeding risk²⁴⁻²⁷ and thus has been discontinued in most institutions. New platelet aggregometers and several new devices are making it easier to assess platelet function.

Platelet Aggregometers

Classical platelet aggregometry using the light transmission principle was developed in the 1960s by Born. This test system measures the increase in light transmission that occurs in direct proportion to platelet aggregation (Figures 42-3 and 42-4) induced by various agonists (e.g., collagen, adenosine diphosphate [ADP], epinephrine) that stimulate different platelet receptors. The test sample is patient platelet-rich plasma (PRP) produced by differential centrifugation of a whole-blood specimen to isolate platelets in plasma with platelet-poor plasma (PPP) as a control. Since its inception, platelet aggregation has been the primary assay to determine alterations in platelet function.

Several newer devices to detect platelet aggregation based on whole-blood impedance, luminescence, and light scatter have since been developed. Table 44-4 compares the newest FDA-cleared platelet aggregometers.

The *Multiplate Analyzer*, also called the *Whole-Blood Multiple Electrode Platelet Aggregometer* (MEA; Dynabyte, Munich, Germany, distributed by DiaPharma Group, West Chester, OH), monitors platelet function by impedance.²⁸ The multiplate has 5 channels with duplicate electrodes per channel. Small whole blood sample volumes are required (300 μ L). The multiplate analysis is based on the principle that upon activation, platelets become sticky and adhere to metal sensor wires (electrodes) (Figure 42-5). The change in electrical resistance between the electrodes is detected and recorded. Three parameters are calculated from each sample: aggregation, area under the curve, and velocity.²⁸ Tests for the therapeutic efficacy of aspirin, clopidogrel, and glycoprotein IIb/IIIa antagonists using arachidonic acid, ADP, and TRAP, respectively, are pending FDA approval. Other applications for platelet function testing are available on this device.

The *AggRAM* (Helena Laboratories Corporation, Beaumont, TX) is a modular system for platelet aggregation and ristocetin cofactor testing that has advanced laser optics utilizing a laser diode measuring at a wavelength of 650 nm to enhance precision of the measured aggregation tracing.²⁹ The *AggRAM* has four channels capable of micro-volume testing, customized result reporting, and internal quality control programs.

The *PAP-8E* from BioData Corporation (Horsham, PA) is an eight-channel platelet aggregometer with a touch screen and on-screen procedure templates (Figure 44-7). It has a programmable pipette and an optional bar code scanner. The *PAP-8E* utilizes light transmission aggregometry and requires a low sample volume (225 μ L). BioData also has a Platelet Function Centrifuge that has been validated for platelet function testing (Figure 44-8).³⁰ PRP is prepared in 30 seconds, and PPP can be prepared in 120 seconds. Platelet-free plasma (PFP) is prepared in 180 seconds.

Chrono-Log Corporation (Havertown, PA) has a *Whole Blood-Optical Lumi-Aggregation System*. The Model 700 aggregometer

TABLE 44-4 A Comparison of Platelet Aggregometers

Parameter	Bio/Data Corp.	ChronoLog Corp.	Helena Laboratories
Instrument name (first year sold)	Platelet Aggregation Profiler, Model PAP-8E (2005)	Whole Blood–Optical Lumi–Aggregation System, Model 700-2/700-4 (2006)	AggRAM (2005)
Operational type	Batch, random access	Batch, random access	Batch, random access
Reagent type	Open reagent system, assay kits, reagents, controls, diluents, buffers	Open reagent system, assay kits, reference plasma, controls	Open reagent system
Operates on whole blood or spun plasma	Spun plasma, optional centrifugation with PDQ to obtain PRP in 3 minutes	Whole blood, spun plasma	Spun plasma
Plasma volume/test	225 μL	225 μL	225 μL
Model type	Benchtop	Benchtop	Benchtop
Number of channels	8	2–4	4–8
Time required for maintenance by lab staff	Weekly: 15 minutes; monthly: 30 minutes	30 minutes when optical calibration required	Daily: 15 minutes; weekly: 15 minutes; monthly: 1 hour

provides platelet aggregation in whole blood or PRP, while simultaneously measuring secretion (Figure 42-6). This aggregometer uses electrical impedance in whole blood and optical density for measuring luminescence. It can be configured as either a two- or four-channel aggregometer.³¹ Either disposable or reusable electrodes can be used for impedance measurements. In addition, Chrono-Log has several other optical and impedance-based instruments. The advantage of whole-blood aggregometry is that it is a

more physiological test due to the ability to measure platelet aggregation in the presence of erythrocytes and leukocytes.

Platelet Function Analyzers

The *PFA-100 Platelet Function Analyzer* (Siemens, Deerfield, IL) is a rapid, automated instrument that is sensitive to quantitative and qualitative platelet abnormalities. Test cartridges contain membranes coated with collagen/epinephrine or collagen/ADP to stimulate platelet aggregation. Whole blood is aspirated under controlled flow conditions through a microscopic aperture



Figure 44-7 PAP-8E from BioData Corporation. An eight-channel aggregometer with a touch screen and a programmable pipet. This instrument requires a sample volume of 225 μL per test and allows for the analysis of routine platelet aggregation testing, measurement of ristocetin cofactor activity, the monitoring of patients with platelet function abnormalities, and the management of antiplatelet therapy. (Photo courtesy of BioData Corp, Horsham, PA.)



Figure 44-8 The PDQ platelet function centrifuge from BioData Corporation is an optional unit to the PAP-8E. The centrifuge is validated for platelet function testing. The PDQ provides platelet-rich plasma, platelet-poor plasma and platelet-free plasma within 5 minutes. (Photo courtesy of BioData Corp, Horsham, PA.)

in the membrane. The time required for a platelet plug to occlude the aperture is an indication of platelet function.^{32,33} The PFA-100 system is successful at detecting von Willebrand disease and the efficacy of aspirin therapy.^{33,34}

The Accumetrics *VerifyNow System* (San Diego, CA) is an optical detection system that measures platelet-induced aggregation by microbead agglutination. The system employs a disposable cartridge that contains lyophilized fibrinogen-coated beads and a platelet agonist specific for the test. Whole blood is dispensed from the blood collection tube into the assay device, with no blood handling required by the operator. The instrument provides an aspirin assay using arachidonic acid as the test reagent, a glycoprotein IIb/IIIa inhibitor (abciximab, tirofiban, eptifibatid) assay using thrombin receptor activation peptide (TRAP) as the test reagent, and a P2Y₁₂ inhibitor (clopidogrel, prasugrel, ticagrelor) assay using ADP as the test reagent.³⁵⁻³⁸

The Plateletworks platelet function assay is available from Helena Laboratories (Beaumont, TX). This assay kit can be run on any standard impedance cell counter found in the hematology laboratory. Aggregation results are based on a platelet count before (high count) and after (lower count) platelet activation using one of the agonist-filled tubes provided in the kit. Testing requires 1 mL of whole blood for baseline count and 1 mL for each additional agonist-containing reagent tube. Results can be obtained in 2 minutes. The *Plateletworks* platelet function kit can be used for presurgical screening and to monitor antiplatelet therapy.³⁹

MOLECULAR COAGULATION TESTING

Molecular testing in the coagulation laboratory is available for patients with thrombophilia (Chapter 39). Molecular testing has become readily available for gene mutations of factor V (FV Leiden) and prothrombin (prothrombin G20210A). Testing for methylene-tetrahydrofolate reductase (MTHFR) is also commonly performed for patients with thrombophilia. However, the clinical utility of MTHFR testing is not clear, and the American College of Medical Genetics and Genomics (ACMG) has recommended that MTHFR testing should not be routinely performed for the workup of patients with thrombophilia.⁴⁰

There are several methods available for the clinical laboratory. Cost and labor usually are considered when evaluating which test system to use in the clinical laboratory. The most common methods used are polymerase chain reaction (PCR)-based assays. PCR is accurate for the detection of both point mutations and single-nucleotide polymorphisms. FV Leiden and prothrombin G20210A, and the MTHFR mutations have been shown to be detectable using this method (Table 44-5). A common method to analyze PCR products is restriction fragment-length polymorphism (RFLP) analysis. However, RFLP analysis is not a high-throughput method and is not suitable for high volume laboratories. Other methods that are PCR-based and some non-PCR-based methods, which no longer require restriction digestion, are also used.⁴¹⁻⁴³ A popular non-PCR based method is the Invader assay which uses allele-specific hybridization in a high-throughput format.

TABLE 44-5 Molecular Techniques for the Evaluation of Hypercoagulable States

Assay	Accuracy	Throughput	Current Clinical Applications
PCR/RFLP	Good	Limited	Factor V Leiden, prothrombin G20210A, MTHFR
PCR/ARMS	Excellent	Intermediate	Factor V Leiden, prothrombin G20210A, MTHFR
Light cycler	Excellent	Intermediate	Factor V Leiden, prothrombin G20210A, MTHFR
Array technology	Excellent	Very high	Factor V Leiden, prothrombin G20210A, MTHFR
Invader assays	Excellent	Limited	Factor V Leiden, prothrombin G20210A, MTHFR
Ligand-based technologies	Excellent	Very high	Factor V Leiden, prothrombin G20210A, MTHFR

MTHFR, Methylene-tetrahydrofolate reductase.

In order to obtain rapid and reliable results, sequence-specific primers, allele-specific oligonucleotides, hybridization, rapid-cycle PCR using LightCycler instrumentation, and nanochips have become available for molecular testing. The major advantages of molecular testing are the increase in sensitivity and specificity and lack of interference by anticoagulants or inhibitors.^{41,42}

Molecular diagnostics in hemophilia and von Willebrand disease are currently limited; however, this is an area that is under development. There is a strong potential for utilization of these assays for the diagnosis and classification of the subgroups of von Willebrand disease. Molecular diagnostics also may have a role in the diagnosis of hemophilia and the mutations involved in both factor VIII and factor IX genes.⁴² Testing for these disorders will become more accessible with the rapid decrease in cost of sequencing and availability of high-throughput next generation sequencing technologies.

The role of molecular diagnostics in thrombophilia workups will continue to grow due to the identification of new genetic mutations and polymorphisms in coagulation disorders. The challenge is for the laboratory to determine which tests to offer and their relevance to patient care.

SELECTION OF COAGULATION INSTRUMENTATION

In today's laboratory, more than ever before, cost effectiveness, testing capabilities, and standardization are top priorities. As an increasing number of tests become available, laboratories must determine what tests to incorporate to provide guidance to physicians in diagnosis and treatment. Identification of testing

needs based on patient population should be the first step in the process. The decisions regarding which tests are the most appropriate for the clinical situations encountered by each laboratory should be made in conjunction with the medical staff. When that input has been obtained, the laboratory can determine the availability and cost of instruments that would meet those requirements.

An instrument should be matched to the anticipated workload. It may not be necessary to purchase a highly sophisticated analyzer capable of performing a large menu of tests if the setting is a small hospital laboratory ordering very few of the more “esoteric” test options available on the instrument under consideration. A batch analyzer with high throughput may be more appropriate for this situation. The option to send out esoteric tests and/or low-volume tests to a reference lab is always available.

Instrument selection criteria may include, but are not limited to, the following:

- Instrument cost
- Consumables cost
- Service response time
- Reliability and downtime
- Maintenance requirements and time
- Operator ease of use
- Breadth of testing menu
- Ability to add new testing protocols
- Reagent lot to lot variation

- Throughput for high-volume testing
- Laboratory information systems (LIS) interface capabilities
- Footprint (the space the instrument occupies; benchtop or floor model)
- Special requirements (water, power, waste drain)
- Flexibility in using other manufacturers’ reagents
- Availability of a training program and continued training support

When the choices have been narrowed based on the most desirable criteria, consideration should be given to additional features. Because no instrument has all the desired features, prioritizing helps the laboratory focus on the capabilities that would be the most advantageous for them. [Box 44-2](#) summarizes several of these specialized features.

CURRENTLY AVAILABLE INSTRUMENTS

A variety of coagulometers address the increasing demand for test volume, random access testing, and test variety. All analyzers perform routine testing quickly and efficiently. The challenge lies in determining which instruments should be considered for a particular laboratory setting and in developing an organized approach for their evaluation. [Table 44-6](#) lists several of the coagulation analyzers currently available, the type of end-point detection offered, and selected specialized features highlighted by the manufacturers in their product information.^{44,45}

BOX 44-2 Specialized Coagulometer Features

Random access: A variety of tests can be performed on a single specimen or multiple specimens in any order as determined by the operator.

Primary tube sampling: Plasma is directly aspirated from an open or capped centrifuged primary collection tube on the analyzer.

Cap piercing: Analyzer aspirates plasma from the closed centrifuged primary collection tube.

Bar coding: Reagents and specimens are identified with a bar code; eliminates manual information entry.

Bidirectional laboratory information system (LIS) interface: Analyzer queries the host computer (LIS) to determine which tests have been ordered. Results are returned to the LIS after verification.

Specimen and instrument flagging: Automated alerts indicate problems with specimen integrity or instrument malfunction.

Liquid level sensing: Operator is alerted when there is inadequate specimen or reagent volume. An alert is also given when the instrument fails to aspirate the required sample volume. Volume is verified each time a specimen or reagent is aspirated.

On-board quality control: Instrument stores and organizes quality control data; may include application of Westgard rules for flagging out-of-range results; instrument may transmit quality control data to the LIS.

Stat capabilities: Operator can interrupt a testing sequence to place a stat specimen next in line for testing.

On-board refrigeration of specimens and reagents: Refrigeration maintains the integrity of specimens and reagents throughout testing and allows reagents to be kept in the analyzer for extended periods, which reduces setup time for less frequently performed tests.

On-board specimen storage capacity: Indicates the number of specimens that can be loaded at a time.

Reflex testing: Instrument can be programmed to perform repeat or additional testing under operator-defined circumstances.

Patient data storage: Test results can be stored for future retrieval; clot formation graphs may be included.

Throughput: Number of tests that can be processed within a specified interval, usually the number of tests per hour; depends on test mix and methodologies.

Total testing (dwell) time: Length of time from specimen placement in the analyzer until testing is completed; depends on the type and complexity of the procedure.

Graph of clot formation: Operator can visualize how the clot is formed over time.

TABLE 44-6 Comparison of the Available Coagulation Analyzers

Instrument Name/Manufacturer	Sample Handling System	FDA-Cleared Clot-Based Tests	FDA-Cleared Chromogenic Tests	FDA-Cleared Immunologic Tests	Methodologies Supported	Number of Different Assays Onboard Simultaneously	Standard Specimen Volume PT/PTT	Detection of Hemolysis/Turbidity	Onboard Patient Dilutions
American Labor Lab A.C.M.	Cuvette, semi automated	PT, PTT, fibrinogen, any clot-based assay	None	None	Clot detection, optical	2	Manual pipetting	No/no	No
Diagnostica Stago STA Satellite	Carousel—primary tube	PT, PTT, fibrinogen	Heparin (UFH, LMWH), AT	D-dimer	Clot detection, mechanical; chromogenic; immunologic	80	5 μ L	No/no (mechanical method)	Yes
Diagnostica Stago STA-R Evolution	Rack with continuous access	PT, PTT, TT, fibrinogen, reptilase, factors, protein C, protein S, lupus anticoagulant, DRVVT screen and confirm	Heparin (UFH, LMWH), protein C, AT, plasminogen, antiplasmin	D-dimer, VWF, total and free protein S, AT antigen	Clot detection, mechanical; chromogenic; immunologic	200	5 μ L	No/no (mechanical method)	Yes
Diagnostica Stago STA Compact, CT	Continuous specimen access—primary tube	PT, PTT, TT, fibrinogen, reptilase, factors, protein C, protein S, lupus anticoagulant, DRVVT	None	None	Clot detection, mechanical	80	5 μ L	No/no (mechanical method)	Yes
Diagnostica Stago Start4 Hemostasis	Manual	PT, PTT, TT, fibrinogen, reptilase, factors, protein C, protein S, lupus anticoagulant	None	None	Clot detection	1	25 μ L	No/no (mechanical method)	No
Diagnostica Stago STA Compact	Continuous specimen access—primary tube	PT, PTT, TT, fibrinogen, reptilase, factors, protein C, protein S, lupus anticoagulant, DRVVT screen and confirm	Heparin (UFH and LMWH), protein C, AT, plasminogen, antiplasmin	D-dimer, VWF, total and free protein S, AT antigen	Clot detection; chromogenic; immunologic	80	50 μ L/5 μ L	No/no (mechanical method)	Yes
Diagnostica Stago STA Compact Plus	Continuous specimen access—primary tube	PT, PTT, TT, fibrinogen, reptilase, factors, protein C, protein S, lupus anticoagulant, DRVVT screen and confirm	Heparin (UFH and LMWH), protein C, AT, plasminogen, antiplasmin	D-dimer, VWF, total and free protein S, AT antigen	Clot detection, mechanical; chromogenic; immunologic	80	50 μ L/5 μ L	No/no (mechanical method)	Yes

Continued

TABLE 44-6 Comparison of the Available Coagulation Analyzers—cont'd

Instrument Name/ Manufacturer	Sample Handling System	FDA-Cleared Clot-Based Tests	FDA-Cleared Chromogenic Tests	FDA-Cleared Immunologic Tests	Methodologies Supported	Number of Different Assays Onboard Simultaneously	Standard Specimen Volume PT/PTT	Detection of Hemolysis/Turbidity	Onboard Patient Dilutions
Diagnostica Stago Destiny Plus	Continuous rack loading	Open system: all clottable assays; PT, PTT, fibrinogen, TT, factors, venom time, protein C, protein S, aPCR, lupus screen and confirm	Open system: all chromogenic assays (protein C, AT FIIa and FXa based, heparin anti-FXa, plasminogen)	Open system: all latex immunoassays (D-dimer)	Clot detection, mechanical and optical (turbidometric); chromogenic; immunologic	10	25 μ L/ 10 μ L	Not necessary	Yes
Diagnostica Stago Destiny Max	Continuous rack loading	Open system: all clottable assays; PT, PTT, fibrinogen, TT, factors, venom time, protein C, protein S, aPCR, lupus screen and confirm	Open system: all chromogenic assays (protein C, AT FIIa and FXa based, heparin anti-FXa, plasminogen)	Open system: all latex immunoassays (D-dimer)	Clot detection, mechanical and optical; chromogenic; immunologic	Unlimited	25 μ L/ 10 μ L	Not necessary	Yes
Helena Laboratories Cascade M-4	Manual	PT, PTT, fibrinogen, TT, factors II, V, VII to XII	None	None	Clot detection, optical, turbidometric	4	100 μ L	No/no	No
Helena Laboratories Cascade M	Manual	PT, PTT, fibrinogen, TT, factors II, V, VII to XII	None	None	Clot detection, optical, turbidometric	1	100 μ L	No/no	No
Instrumentation Laboratory ACL 300	Continuous rack loading	PT, PTT, fibrinogen, TT, factors, FVIII (with VWF)		D-dimer HS		500		No/no	Yes
Instrumentation Laboratory ACL 500	Continuous rack loading	PT, PTT, fibrinogen, TT, factors, lupus (SCT and DRVVT), protein C, protein S, aPCR-V, FVIII (with VWF)	Heparin anti-FXa, protein C, AT, plasminogen, antiplasmin	D-dimer, D-dimer HS, VWF (activity and antigen), free protein S, FXIII antigen, homocysteine	Clot detection, LED optical; chromogenic; immunologic (turbidometric)	500	PT and PTT: 50 μ L; FVIII: 25 μ L	No/no	Yes
Instrumentation Laboratory ACL 700	Continuous rack loading	PT, PTT, fibrinogen, TT, factors, lupus (SCT and DRVVT), aPCR-V, protein C, protein S, FVIII (with VWF)	Heparin anti-FXa, protein C, AT, plasminogen, antiplasmin	D-dimer, D-dimer HS, VWF (activity and antigen), free protein S, FXIII antigen, homocysteine	Clot detection, LED optical; chromogenic; immunologic	500	PT and PTT: 50 μ L; FVIII: 25 μ L	No/no	Yes

TABLE 44-6 Comparison of the Available Coagulation Analyzers—cont'd

Instrument Name/ Manufacturer	Sample Handling System	FDA-Cleared Clot-Based Tests	FDA-Cleared Chromogenic Tests	FDA-Cleared Immunologic Tests	Methodologies Supported	Number of Different Assays Onboard Simultaneously	Standard Specimen Volume PT/PTT	Detection of Hemolysis/Turbidity	Onboard Patient Dilutions
Instrumentation Laboratory ACL Elite Series	Tray-primary tubes	PT, PTT, fibrinogen, TT, factors, protein C, protein S, lupus (SCT and DRVT), aPCR-V	Heparin anti-FXa, protein C, AT, plasminogen, antiplasmin, FVIII	D-dimer, VWF (activity and antigen), free protein S, FXIII antigen, homocysteine	Clot detection, LED optical (nephelometric); chromogenic; immunologic	22	PT and PTT: 60 μ L; FVIII: 18 μ L	No/no	Yes
Siemens BFT II	Manual	PT, PTT, fibrinogen	None	None	Turbodensitometric	1	50 μ L	No/no	No
Siemens CA-1500	10-Tube position sample rack \times 5	PT, PTT, fibrinogen, TT, reptilase time, factors, DRVT screen and confirm, FV Leiden, protein C clot, protein S activity	Innovance AT, Berichrom AT, plasminogen, FVIII chromogenic, anti-plasmin, protein C chromogenic, heparin	Innovance D-dimer	Clot detection, optical, turbidometric; chromogenic; immunologic	15	50 μ L/5 μ L	No/yes	Yes
Siemens CA-7000	Rack	PT, PTT, fibrinogen, TT, reptilase time, factors, DRVT screen and confirm, FV Leiden, protein C clot, protein S activity	Innovance AT, Berichrom AT, plasminogen, FVIII chromogenic, anti-plasmin, protein C chromogenic, heparin	Innovance D-dimer	Clot detection, optical, turbidometric; chromogenic; immunologic	20	50 μ L/5 μ L	No/yes	Yes
Siemens BCS XP	10-Tube position sample rack	PT, PTT, fibrinogen, TT, reptilase time, factors, DRVT screen and confirm, FV Leiden, protein C clot, protein S activity	Innovance AT, Berichrom AT, plasminogen, FVIII chromogenic, anti-plasmin, protein C chromogenic, heparin	Innovance D-dimer	Clot detection, optical (xenon flasher lamp); chromogenic; immunologic	>100	50 μ L/20 μ L min. 100 μ L (includes dead volume)/ 50 μ L	Yes/no	Yes
Siemens CA-600	10-Tube position sample rack	PT, PTT, fibrinogen, TT, reptilase time, protein C clot, factor assays	Innovance AT, Berichrom AT, protein C chromogenic, heparin	Innovance D-dimer	Clot detection, optical; turbidometric; chromogenic; immunologic	5	50 μ L/5 μ L	No/yes	Yes
LABiTech GmbH CoaData 2004	Semiautomated manual pipette-auto start	PT, PTT	None	None	Clot detection, optical; turbodensitometric	1	50 μ L	No/no	No

aPCR, Activated protein C resistance; AT, antithrombin; DRVT, dilute Russell viper venom test; F, factor; LMWH, low molecular weight heparin; HS, high sensitivity; PT, prothrombin time; PTT, partial thromboplastin time; SCT, silica clotting test; TT, thrombin clotting time; UFH, unfractionated heparin; VWF, von Willebrand factor.

SUMMARY

- Advanced technology used in semiautomated and automated analyzers has greatly improved coagulation testing accuracy and precision.
- End-point detection methodologies employed by modern coagulation analyzers include mechanical, photo-optical, nephelometric, chromogenic, and immunologic methods.
- Advances in end-point detection methodologies have greatly expanded the testing capabilities available in the routine coagulation laboratory.
- Markedly improved instrument precision and reduced reagent volume requirements have led to substantial cost savings in coagulation testing.
- Instrument manufacturers have incorporated many features that have enhanced efficiency, safety, and diagnostic capabilities in hemostasis testing.
- Coagulation analyzer flagging alert functions warn the operator when sample or instrument conditions exist that might lead to invalid test results so that appropriate actions can be taken to ensure test accuracy.
- Each method of end-point detection has advantages and disadvantages that must be recognized and understood to ensure the validity of test results.
- Several methods to evaluate platelet function are available for both general platelet function testing and antiplatelet drug monitoring.
- The role of molecular diagnostics will continue to grow to identify new mutations and polymorphisms associated with bleeding and clotting disorders.
- Coagulation testing has been incorporated into the arena of point-of-care testing primarily to enhance the patient's and physician's ability to monitor oral anticoagulant therapy.
- A systematic approach to the evaluation and selection of a new coagulation analyzer should be developed and followed to determine the best instrument for a specific laboratory setting.

Now that you have completed this chapter, go back and read again the case study at the beginning and respond to the questions presented.

REVIEW QUESTIONS

Answers can be found in the Appendix.

1. The photo-optical method of end-point detection can be described as:
 - a. Measurement of a color-producing chromophore at a wavelength of 405 nm
 - b. Measurement of the change in OD of a test solution as a result of fibrin formation
 - c. Application of an electromagnetic field to the test cuvette to detect the decreased motion of an iron ball within the cuvette
 - d. Measurement of the turbidity of a test solution resulting from the formation of antigen-antibody complexes using latex particles
2. Modern coagulation analyzers have greatly enhanced the ability to perform coagulation testing as a result of which of the following?
 - a. Maintenance of a level of accuracy and precision similar to that of manual methods
 - b. Increase in reagent volume capabilities to improve sensitivity
 - c. Automatic adjustment of results for interfering substances
 - d. Improved flagging capabilities to identify problems in sample quality or instrument function
3. Which of the following is considered to be an advantage of the mechanical end-point detection methodology?
 - a. It is not affected by lipemia in the test sample
 - b. It has the ability to provide a graph of clot formation
 - c. It can incorporate multiple wavelengths into a single testing sequence
 - d. It can measure proteins that do not have fibrin formation as the end-point
4. Which of the following methods use the principle of changes in light scatter or transmission to detect the end-point of the reaction?
 - a. Immunologic, mechanical, photo-optical
 - b. Photo-optical, nephelometric, mechanical
 - c. Photo-optical, nephelometric, immunologic
 - d. Chromogenic, immunologic, mechanical
5. Which of the following is a feature of semiautomated coagulation testing analyzers?
 - a. The temperature is maintained externally by a heat block or water bath
 - b. Reagents and samples usually are added manually by the operator
 - c. Timers are automatically started as soon as the analyzer adds reagents to the test cuvette
 - d. The end-point must be detected by the operator

6. When a sample has been flagged as being icteric by an automated coagulation analyzer, which method would be most susceptible to erroneous results because of the interfering substance?
 - a. Mechanical clot detection
 - b. Immunologic antigen-antibody reaction detection
 - c. Photo-optical clot detection
 - d. Chromogenic end-point detection
7. Platelet function testing has been incorporated into the routine coagulation laboratory in recent years as a result of:
 - a. Increased use of drugs that stimulate platelet production in patients receiving chemotherapy
 - b. The convenience of being able to do the testing on the same instrument that performs the coagulation testing
 - c. Increased therapeutic use of aspirin in the treatment of heart disease
 - d. Increased outpatient/outreach testing that prevents the laboratory from having access to patients to do bleeding time tests
8. All of the following are performance characteristics to consider in the selection of a coagulation analyzer *except*:
 - a. Location of the manufacturer's home office
 - b. Instrument footprint
 - c. Ease of use for the operator
 - d. Variety of tests the instrument can perform
9. The PFA-100 measures platelet function by:
 - a. Detecting the change in blood flow pressure along a small tube when a clot impairs blood flow
 - b. Detecting the aggregation of latex beads coated with platelet activators
 - c. Graphing the transmittance of light through platelet-rich plasma over time after addition of platelet activators
 - d. Detecting the time it takes for a clot to form as blood flows through a small aperture in a tube coated with platelet activators
10. Point-of-care coagulation testing is used mainly:
 - a. To monitor patients receiving oral anticoagulant therapy
 - b. To monitor patients taking platelet inhibitors such as aspirin
 - c. To provide a baseline for all subsequent patient test result comparisons when the patient starts any kind of anticoagulant therapy
 - d. To monitor obstetric patients at risk of fetal loss

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Pediatric and Geriatric Hematology and Hemostasis

Linda H. Goossen

OUTLINE

Pediatric Hematology and Hemostasis

Prenatal Hematopoiesis

Hematopoiesis of the Newborn

Pediatric Developmental Stages

Gestational Age and Birth Weight

Red Blood Cell Values at Birth

White Blood Cell Values in the Newborn

Platelet Values in the Newborn

Neonatal Hemostasis

Geriatric Hematology and Hemostasis

Aging and Hematopoiesis

Assessment of Hematologic Parameters in Healthy Elderly Adults

Anemia and the Elderly

Hematologic Neoplasia in Older Individuals

OBJECTIVES

After completion of this chapter, the reader will be able to:

- Describe the major differences in reference intervals for the complete blood count, reticulocyte count, and nucleated red blood cells (NRBCs) in preterm newborns, full-term newborns, infants, children, adults, and elderly adults.
- Explain the cause of physiologic anemia of infancy and the time frame in which it is expected.
- Describe normal RBC morphology in neonates.
- Compare the RBC survival in preterm and full-term infants with that in adults.
- Recognize and list factors affecting sample collection that can have an impact on the interpretation of hematology values in newborns.
- Compare and contrast the morphology of lymphocytes in children and in adults, and indicate reasons for differences.
- Describe the general association between age and hemoglobin levels in the elderly.
- Explain the clinical significance of anemia in the elderly.
- Name the two most common anemias seen in the elderly and their common causes in this age group.
- List other anemias affecting elderly individuals.
- Compare the frequency of acute lymphoblastic leukemias and chronic lymphocytic leukemias in children and the elderly.
- Compare the hemostatic systems of newborns, children, adults, and the elderly, including the risk of bleeding and thrombosis.
- Name hematologic malignancies that are more common in the elderly than in other age groups.

CASE STUDY

After studying this chapter, the reader should be able to respond to the following case study:

A full-term newborn infant in no apparent distress had a complete blood count (CBC) performed as part of a panel of testing for infants born to mothers who received no prenatal care.

Results were as follows:

	Patient Results	Reference Intervals
WBCs ($\times 10^9/L$)	18.0	9.0–37.0
RBCs ($\times 10^{12}/L$)	5.28	4.10–6.10
HGB (g/dL)	18.5	16.5–21.5
HCT (%)	55.5	48–68
MCV (fL)	105.1	95–125

WBC differential: 50% neutrophils and 50% lymphocytes

RBC morphology: Macrocytic with slight to moderate polychromasia.

There were 7 NRBCs/100 WBCs.

- Are the results for hemoglobin and hematocrit within the reference intervals expected for a newborn?
- What is the significance of the elevated MCV, NRBCs, and polychromasia?
- Comment on the WBC and differential count.

Hematologic values are fairly stable throughout adult life, but significant differences exist in the pediatric and, to some extent, in the geriatric populations. This chapter focuses on the more significant differences.

PEDIATRIC HEMATOLOGY AND HEMOSTASIS

Children are not merely “small adults.” The newborn infant, older child, and adult exhibit profound hematologic differences from one another. Because children mature at different rates, it is inappropriate to use adult reference intervals for the assessment of pediatric blood values. Historically, pediatric reference intervals were inferentially established from adult data because of the limitations in attaining analyzable data. Pediatric procedures required large blood draws and tedious methodologies and lacked standardization. The implementation of child-friendly phlebotomy techniques and micropediatric procedures has revolutionized laboratory testing. Pediatric hematology has emerged as a specialized science with age-specific reference intervals that correlate with the hematopoietic, immunologic, and chemical changes in a developing child.

Dramatic changes occur in the blood and bone marrow of the newborn infant during the first hours and days after birth, and there are rapid fluctuations in the quantities of all hematologic elements. Significant hematologic differences are seen between term and preterm infants and among newborns, infants, young children, and older children. This chapter reviews neonatal hematopoiesis, which is discussed in detail in Chapter 7, as a prerequisite to understanding the changes in pediatric hematologic reference intervals, morphologic features, and age-specific physiology.

Prenatal Hematopoiesis

Hematopoiesis, the formation and development of blood cells from hematopoietic stem cells, begins in the first weeks of embryonic development and proceeds systematically through three phases of development: mesoblastic (yolk sac), hepatic (liver), and myeloid (bone marrow). The first cells produced in the developing embryo are primitive erythroblasts formed in the yolk sac. These cells appear megaloblastic and circulate as large nucleated cells, synthesizing embryonic hemoglobins. A second wave of yolk-sac derived erythroid progenitor cells, termed *burst forming units–erythroid* (BFU-E), appear around four weeks and are thought to seed the fetal liver.¹⁻⁶

By the second month of gestation, hematopoiesis ceases in the yolk sac, and the liver becomes the center for hematopoiesis, reaching its peak activity during the third and fourth gestational months. Leukocytes of each cell type systematically make their appearance. In week 9 of gestation, lymphocytes can be detected in the region of the thymus. They are subsequently found in the spleen and lymph nodes. During the fourth and fifth gestational months, the bone marrow emerges as a major site of blood cell production, and it becomes the primary site by birth (Chapter 7).¹⁻⁶

Hematopoiesis of the Newborn

Hematopoietically active bone marrow is referred to as *red marrow*, as opposed to inactive *yellow* (fatty) *marrow*. At the time of

birth, the bone marrow is fully active and almost completely cellular, with all hematopoietic cell lineages undergoing cellular differentiation and amplification. In addition to the mature cells in fetal blood, there are significant numbers of circulating progenitor cells in cord blood.^{7,8}

In a full-term infant, hepatic hematopoiesis has ceased except in widely scattered small foci that become inactive soon after birth.¹⁻⁶ Postembryonic extramedullary hematopoiesis is abnormal in a full-term infant. In a premature infant, foci of hematopoiesis are frequently seen in the liver and occasionally observed in the spleen, lymph nodes, or thymus.¹⁻¹⁰

Pediatric Developmental Stages

Pediatric hematologic values change markedly in the first weeks and months of life, and many variables influence the interpretation of what might be considered healthy at the time of birth. Thus it is important to provide age-appropriate pediatric hematology reference intervals that extend from neonatal life through adolescence. The pediatric population can be categorized with reference to three different developmental stages: the neonatal period, which represents the first 4 weeks of life; infancy, which incorporates the first year of life; and childhood, which spans age 1 to puberty (age 8 to 12 years).

Preterm, low-birth-weight infants are more apt to develop health problems than are other newborns. Since the 1970s, the rising quality of medical care in neonatal intensive care units in the United States and other industrialized countries has improved markedly the survival of smaller infants born at younger gestational ages with less mature hematopoietic systems.

Gestational Age and Birth Weight

Hematologic values obtained from full-term infants generally do not apply to preterm infants, and laboratory values for low-birth-weight preterm infants differ from values for extremely low-birth-weight infants. A full-term infant is defined as an infant who has completed 37 to 42 weeks of gestation. Infants born before 37 weeks' gestation are referred to as *premature* or *preterm*, whereas infants delivered after 42 weeks are considered *postterm*.^{11,12} Infants can be subcategorized further by birth weight as (1) appropriate size for gestational age; (2) small for gestational age, including low-birth-weight infants (2500 g or less); (3) very low-birth-weight infants (1500 g or less); (4) extremely low-birth-weight micropreemies (1000 g or less); and (5) large for gestational age (more than 4000 g).^{11,12}

Red Blood Cell Values at Birth

Neonatal hematologic values are affected by the gestational age of the infant, birth weight, the age in hours after delivery, the presence of illness, and the level of support required. Other important variables to be considered when evaluating laboratory data include site of sampling and technique (capillary versus venous puncture, warm or unwarmed extremity), timing of sampling, and conditions such as the course of labor and the treatment of the umbilical vessels, and maternal drug use.^{1,2,13} The presence of fetal hemoglobin (Hb F), bilirubin, and lipids in newborns can also interfere with hematology

laboratory testing.¹⁴⁻¹⁷ As with all laboratory testing, each laboratory should establish reference intervals based on its instrumentation, methods, and patient population (Chapter 5).

Red Blood Cell Count

Refer to the inside front cover of this book for red blood cell (RBC) reference intervals. The RBC count increases during the first 24 hours of life, remains at this plateau for about 2 weeks, and then slowly declines. This “polycythemia” of the newborn¹⁸ may be explained by in utero hypoxia, which becomes more pronounced as the fetus grows. Hypoxia, the trigger for increased secretion of erythropoietin, stimulates erythropoiesis.¹⁹ At birth, the physiologic environment changes, and the fetus makes the transition from its placenta-dependent oxygenation to the increased tissue oxygenation by the lungs. This increased oxygen tension suppresses erythropoietin production, which is followed by a decrease in RBC and hemoglobin production. Studies show that erythropoietin levels before birth are equal to or greater than adult levels with a gradual drop to near zero a few weeks after birth.²⁰⁻²⁴ This decline corresponds to the physiologic anemia seen at 5 to 8 weeks of life, with the RBCs reaching their lowest count at 7 weeks of age and hemoglobin reaching its lowest concentration at 9 weeks of age.¹⁹

Erythrocyte Morphology of the Neonate. Early normoblasts are megaloblastic, hypochromic, and irregularly shaped. During hepatic hematopoiesis, normoblasts are smaller than the megaloblasts of the yolk sac but are still macrocytic. Erythrocytes remain macrocytic from the first 11 weeks of gestation until day 5 of postnatal life (Figure 45-1).^{1,2,13,20,21,24,25}

The macrocytic RBC morphology gradually changes to the characteristic normocytic, normochromic morphology. Orthochromic normoblasts frequently are observed in the full-term infant on the first day of life but disappear within postnatal days 3 to 5. These nucleated RBCs (NRBCs) may persist longer than a week in immature infants. The average number of

NRBCs ranges from 3 to 10 per 100 white blood cells (WBCs) in a healthy full-term infant to 25 NRBCs per 100 WBCs in a premature infant. The presence of NRBCs for more than 5 days suggests hemolysis, hypoxic stress, or acute infection.^{1,2,6,13,20-24}

The erythrocytes of newborns show additional morphologic differences. The number of biconcave discs relative to stomatocytes is reduced in neonates (43% discs, 40% stomatocytes) compared with adults (78% discs, 18% stomatocytes).²⁶ In addition, increased numbers of pitted cells, burr cells, spherocytes, and other abnormally shaped erythrocytes are seen in neonates. The number of these “dysmorphic” cells is even higher in premature infants. Zipursky and colleagues found 40% discs, 30% stomatocytes, and 27% additional poikilocytes in premature infants.^{1,26}

Reticulocyte Count

An apparent reticulocytosis exists during gestation, decreasing from 90% reticulocytes at 12 weeks’ gestation to 15% at 6 months’ gestation and ultimately to 4% to 6% at birth. Reticulocytosis persists for about 3 days after birth and then declines abruptly to 0.8% reticulocytes on postnatal days 4 to 7. At 2 months, the number of reticulocytes increases slightly, followed by a slight decline from 3 months to 2 years, when adult levels of 0.5% to 2.5% are attained.^{1,2,6,13,20-24} The reticulocyte count of premature infants is typically higher than that of term infants; however, the count can vary dramatically, depending on the extent of illness in the newborn. Significant polychromasia seen on a Wright-stained blood film is indicative of postnatal reticulocytosis (Figure 45-2).

Hemoglobin

Full-Term Infants. Hemoglobin synthesis results from an orderly evolution of a series of embryonic, fetal, and adult hemoglobins. At birth Hb F constitutes 60% to 90% of the total hemoglobin.²⁷ Hb F declines from 90% to 95% at 30 weeks’ gestation to approximately 7% at 12 weeks after birth and stabilizes at $3.2 \pm 2.1\%$ at 16 to 20 weeks after birth.²⁸ The switch

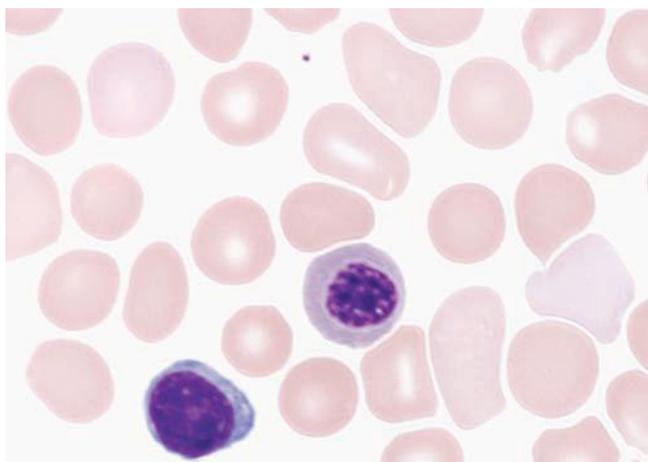


Figure 45-1 Peripheral blood from a healthy newborn demonstrating a normal lymphocyte, macrocytes, polychromasia, and one nucleated red blood cell ($\times 1000$).

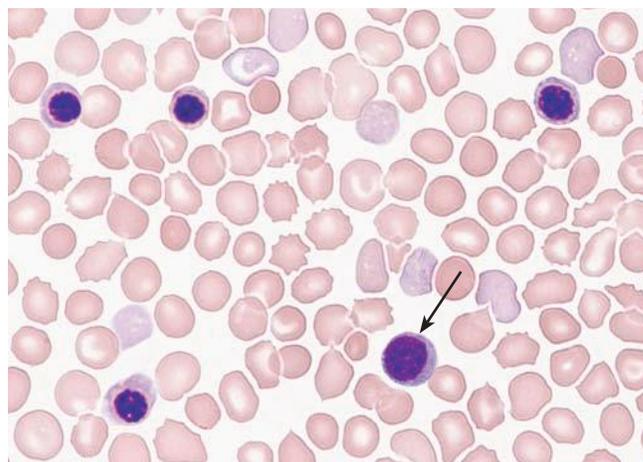


Figure 45-2 Peripheral blood film from a premature infant showing a normal lymphocyte (arrow), four nucleated red blood cells, and increased polychromasia ($\times 500$).

from Hb F to Hb A is genetically controlled and determined by gestational age; it does not appear to be influenced by the age at which birth occurs.^{24,29} Chapter 10 provides an in-depth discussion of the ontogeny, structure, and types of hemoglobin.

The concentration of hemoglobin fluctuates dramatically in the weeks and months after birth as a result of physiologic changes, and various factors must be considered when analyzing pediatric hematologic values. The site of sampling, gestational age, and time interval between delivery and clamping of the umbilical cord can influence the hemoglobin level in newborn infants.^{1,2,6,9-11} In addition, there are significant differences between capillary and venous blood hemoglobin levels. Capillary samples in newborns generally have a higher hemoglobin concentration than venous samples, which can be attributed to circulatory factors.^{14,20-22,24,25} Racial differences must also be considered when evaluating hemoglobin levels in children. Black children have hemoglobin levels averaging 0.5 g/dL lower than those in white children.³⁰

The reference interval for hemoglobin for a full-term infant at birth is 16.5 to 21.5 g/dL; levels less than 14 g/dL are considered abnormal.^{2,20,21} The average hemoglobin value for a preterm infant who is small for gestational age is 17.1 g/dL, lower than that for a full-term infant; hemoglobin values less than 13.7 g/dL are considered abnormal in preterm infants.^{20,21}

Physiologic Anemia of the Neonate. The hemoglobin concentration of term infants decreases during the first 5 to 8 weeks of life, a condition known as *physiologic anemia of infancy*. Infants born prematurely also experience a decrease in hemoglobin concentration, which is termed *physiologic anemia of prematurity*.^{2,32-34} Along with a decrease in hemoglobin, there is a reduction in the number of RBCs, a decrease in the reticulocyte percentages (Table 45-1), and undetectable levels of erythropoietin associated with the transition from the placenta to the lungs as a source of oxygen. When the hemoglobin concentration decreases to approximately 11 g/dL, erythropoietic activity increases until it reaches its adult levels by 14 years of age.^{18,35-40} Also contributing to the physiologic anemia is the shortened life span of the fetal RBC. Studies of chromium-labeled newborn RBCs estimate a survival time of 60 to 70 days, with correction for the elution rate of chromium from

newborn cells.¹ The life span of RBCs in premature infants is about 35 to 50 days.¹ The more immature the infant, the greater the degree of reduction.^{1,21,24} The reasons for the shortened life span of the erythrocytes are unclear. This physiologic anemia is not known to be associated with any abnormalities in the infant.

The hemoglobin levels of premature infants are typically 1 g/dL or more below the values of full-term infants. Thereafter, a gradual recovery occurs, which results in values approximating those of healthy full-term infants by about 1 year of age.^{15,18,23,35,36,40} Very low-birth-weight infants (less than 1500 g) show a progressive decline in hemoglobin, RBC count, mean cell volume (MCV), mean cell hemoglobin (MCH), and mean cell hemoglobin concentration (MCHC) and have a slower recovery than other preterm and term infants.

Hematocrit

The average capillary hematocrit (HCT) at birth for healthy full-term infants is 61% (reference interval, 48% to 68%).^{1,2,24} Frequently, newborns with increased hematocrits, especially values greater than 65%, experience hyperviscosity of the blood. This can cause problems in producing a high-quality peripheral blood film.

The hematocrit usually increases approximately 5% during the first 48 postnatal hours; this is followed by a slow linear decline to 46% to 62% at 2 weeks and 32% to 40% between the second and fourth months.^{1,2,25} Adult values of 47% for males and 42% for females are achieved during adolescence. Very low-birth-weight preterm infants are frequently anemic at birth (Table 45-1). Many require transfusions or erythropoietin injections or both.

Red Blood Cell Indices

The RBC indices and RBC distribution width (RDW) provide one means for assessing the type of anemia (Chapter 19).

Mean Cell Volume. The erythrocytes of newborn infants are markedly macrocytic at birth. The average MCV for full-term infants is 119 ± 9.4 fL; however, a sharp decrease occurs during the first 24 hours of life.^{1,2} The MCV continues to decrease to 90 ± 12 fL in 3 to 4 months.^{2,18,37} The more premature the infant, the higher the MCV. A newborn with an MCV of less than 94 fL should be evaluated for α -thalassemia or iron deficiency.^{1,41}

Mean Cell Hemoglobin. MCH is 30 to 42 pg in healthy neonates and 27 to 41 pg in premature infants.^{18,37}

Mean Cell Hemoglobin Concentration. The average MCHC is the same for full-term infants, premature infants, and adults: approximately 33 g/dL.

Red Blood Cell Distribution Width. The red blood cell distribution width is elevated in newborns, with a reference interval of 14.2% to 17.8% the first 30 days of life. After that it gradually decreases and reaches the adult reference interval by 6 months of age.²⁵

TABLE 45-1 Hematologic Values for Very Low-Birth-Weight Infants During the First 6 Weeks of Life

Hematologic Value	AGE OF INFANT (DAYS)			
	3	12-14	24-26	40-42
Hemoglobin (g/dL)	15.6	14.4	12.4	10.6
Hematocrit (%)	47	44	39	33
Red blood cells ($\times 10^{12}/L$)	4.2	4.1	3.8	3.4
Reticulocytes (%)	7.1	1.7	1.5	1.8
Platelets ($\times 10^9/L$)	203.5	318	338	357
White blood cells ($\times 10^9/L$)	9.5	12.3	10.4	9.1

Modified from Obladen M, Diepold K, Maier RF, et al: Venous and arterial hematologic profiles of very low birth weight infants, *Pediatrics* 106:707-711, 2000.

TABLE 45-2 Leukocyte Count Reference Intervals by Age*

Age	NEUTROPHILS			LYMPHOCYTES			EOSINOPHILS		MONOCYTES		TOTAL WBC COUNT	
	Mean	Range	%	Mean	Range	%	Mean	%	Mean	%	Mean	Range
Birth	4.0	2.0–6.0	—	4.2	2.0–7.3	—	0.1	—	0.6	—	— [‡]	—
12 hr	11.0	7.8–14.5	—	4.2	2.0–7.3	—	0.1	—	0.6	—	—	—
24 hr	9.0	7.0–12.0	—	4.2	2.0–7.3	—	0.1	—	0.6	—	—	—
1–4 wk	3.6	1.8–5.4	—	5.6	2.9–9.1	—	0.2	—	0.7	—	—	—
6 mo	3.8	1.0–8.5	32	7.3	4.0–13.5	61	0.3	3	0.6	5	11.9	6.0–17.5
1 yr	3.5	1.5–8.5	31	7.0	4.0–10.5	61	0.3	3	0.6	5	11.4	6.0–17.5
2 yr	3.5	1.5–8.5	33	6.3	3.0–9.5	59	0.3	3	0.5	5	10.6	6.0–17.0
4 yr	3.8	1.5–8.5	42	4.5	2.0–8.0	50	0.3	3	0.5	5	9.1	5.5–15.5
6 yr	4.3	1.5–8.0	51	3.5	1.5–7.0	42	0.2	3	0.4	5	8.5	5.0–14.5
8 yr	4.4	1.5–8.0	53	3.3	1.5–6.8	39	0.2	2	0.4	4	8.3	4.5–13.5
10 yr	4.4	1.8–8.0	54	3.1	1.5–6.5	38	0.2	2	0.4	4	8.1	4.5–13.5
16 yr	4.4	1.8–8.0	57	2.8	1.2–5.2	35	0.2	3	0.4	5	7.8	4.5–13.0

Data from Cairo MS, Bracho F: White blood cells. In Rudolph CD, Rudolph AM, Hostetter MK, et al, editors: *Rudolph's pediatrics*, ed 21, New York, 2003, McGraw-Hill, p 1548.

*Numbers of leukocytes are $\times 10^9/L$ (or thousands per microliter), ranges are estimates of 95% confidence limits, and percentages refer to differential counts.

[†]Neutrophils include band cells at wall ages and a small number of metamyelocytes and myelocytes in the first few days of life. WBC, white blood cell.

[‡]Dashes indicate insufficient data for a reliable estimate.

Anemia in Infants and Children

Nutritional deficiencies in infants and children can result in iron deficiency anemia and, rarely, in megaloblastic anemia (Chapters 20 and 21), particularly in low-birth-weight and premature infants. These anemias are associated with abnormal psychomotor development; however, they can easily be treated with dietary fortification.⁴²⁻⁴⁶

Iron Deficiency Anemia. Iron deficiency anemia is the most common pediatric hematologic disorder and the most frequent cause of anemia in childhood.⁴⁷ The occurrence of iron deficiency anemia in infants has decreased in the United States due to iron fortification of infant formula and increased rates of breastfeeding.⁴⁸ However, the prevalence is still 2% in toddlers 1 to 2 years of age and 3% in children 3 to 5 years of age⁴⁹ and is related to early introduction and excessive intake of whole cow's milk.^{42,50} Chapter 20 provides an in-depth discussion of iron deficiency anemia.

Ancillary Tests for Anemia in Infants and Children. The differential diagnosis of anemia in infants and children relies on a variety of ancillary tests. The reference intervals for a number of these tests differ from those for adults. Haptoglobin levels are so low as to be undetectable in neonates, which makes it unreliable as a marker of infant hemolysis.⁵¹ Transferrin levels are also lower in neonates, increasing rapidly after birth and reaching adult levels at 6 months.⁵¹ Both serum ferritin and serum iron are high at birth, rise during the first month, drop to their lowest level between 6 months and 4 years of age, and remain low throughout childhood.⁵²⁻⁵⁴ Consideration of these differences is important when interpreting hematology laboratory results for infants and children.

White Blood Cell Values in the Newborn

Fluctuations in the number of WBCs are common at all ages but are greatest in infants (Table 45-2). Leukocytosis is typical at birth for full-term and preterm infants, with a wide reference interval.¹⁸ There is an excess of segmented neutrophils and bands, and an occasional metamyelocyte, with no evidence of disease. The absolute neutrophil count rises within the first 8 to 12 hours following birth and then declines by 12 hours to a constant level.^{2,18,55,56}

Neutrophilic Leukocytes

Refer to Table 45-2 and the inside front cover for the leukocyte reference intervals for healthy full-term infants. Term and premature infants show a greater absolute neutrophil count than that found in older children, who characteristically maintain a predominance of lymphocytes. Band forms are also higher for the first 3 to 4 days after birth (Table 45-3).

TABLE 45-3 Neutrophil and Band Counts for Newborns During the First 2 Days of Life*

Age	Absolute Neutrophil Count ($\times 10^9/L$)	Absolute Band Count ($\times 10^9/L$)
Birth	3.5–6.0	1.3
12 hr	8.0–15.0	1.3
24 hr	7.0–13.0	1.3
36 hr	5.0–9.0	0.7
48 hr	3.5–5.2	0.7

Modified from Luchtman-Jones L, Wilson DB: The blood and hematopoietic system. In Martin RJ, Fanaroff AA, Walsh MC, editors: *Fanaroff and Martin's neonatal-perinatal medicine*, ed 9, Philadelphia, 2011, Elsevier Mosby, p 1325.

*Reference intervals were obtained from the assessment of 3100 separate white blood cell counts obtained from 965 infants; 513 counts were from infants considered to be completely healthy at the time the count was obtained and for the preceding and subsequent 48 hours. There was no difference in the reference intervals when values were stratified by infant birth weight or gestational age.

Newborn females have absolute neutrophil counts averaging 2000 cells/ μL higher than those of males; neonates whose mothers have undergone labor have higher counts than neonates delivered by cesarean section with no preceding maternal labor.^{55,56} There is some evidence that absolute neutrophil counts are lower in healthy black children than in white children.^{57,58}

Premature Infants. At birth, preterm infants exhibit a left shift, with promyelocytes and myelocytes frequently observed. The trend to lymphocyte predominance occurs later than in full-term infants. The neutrophil counts in premature infants are similar to or slightly lower than the neutrophil counts in full-term infants during the first 5 days of life; however, the count gradually declines to $2.5 \times 10^9/\text{L}$ (1.1 to $6.0 \times 10^9/\text{L}$) at 4 weeks.⁵⁹ There is no significant difference in the absolute neutrophil count of infants by birth weight or gestational age; however, very low-birth-weight infants have a significantly lower limit ($1.0 \times 10^9/\text{L}$) compared with larger infants.⁵⁹⁻⁶¹

Neutropenia. Neutropenia is defined as a reduction in the number of circulating neutrophils to less than $1.5 \times 10^9/\text{L}$. Neutropenia accompanied by bands and metamyelocytes is often associated with infection, particularly in preterm neonates. Neutropenia represents a decrease in neutrophil production or an increase in consumption.⁶²

Neutrophilia. Neutrophilia refers to an increase in the absolute number of neutrophils to greater than $8.0 \times 10^9/\text{L}$. Morphologic changes associated with infection include Döhle bodies, vacuoles, and toxic granulation.⁶³

Eosinophils and Basophils

The percentages of eosinophils and basophils remain consistent throughout infancy and childhood. Refer to the inside front cover of this book for reference intervals.

Lymphocytes

Lymphocytes constitute about 30% of the leukocytes at birth and increase to 60% at 4 to 6 months. They decrease to 50% by 4 years, to 40% by 6 years, and to 30% by 8 years.^{13,20} Benign immature B cells (hematogones), although predominantly found in the bone marrow, can sometimes be seen in the peripheral blood of newborns. These lymphocytes are primarily mid-stage B cells^{64,65} and are frequently referred to as “baby” or “kiddie” lymphocytes. They vary in diameter from 10 to 20 μm , have scant cytoplasm and condensed but homogeneous nuclear chromatin, and may have small, indistinct nucleoli (Figures 45-3 and 45-4).⁶⁶⁻⁶⁸ Although these lymphocytes may be similar in appearance to the malignant cells seen in childhood acute lymphoblastic leukemia (ALL), these benign cells lack the asynchronous or aberrant antigen expression seen in ALL and thus can be differentiated from the lymphocytes of infant ALL by immunophenotyping (Chapter 32).^{69,70}

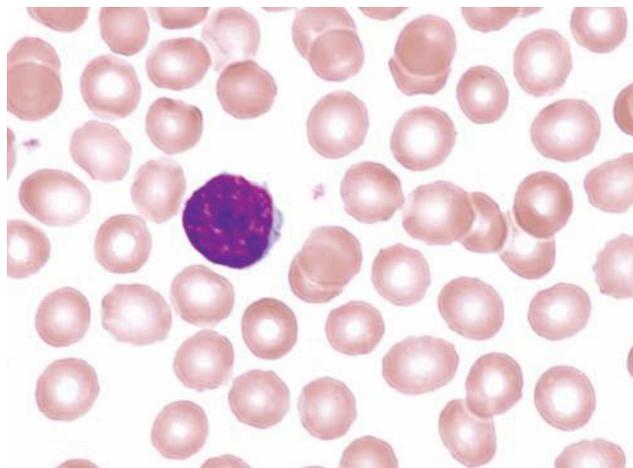


Figure 45-3 Peripheral blood film from a healthy newborn showing a benign lymphocyte ($\times 1000$).

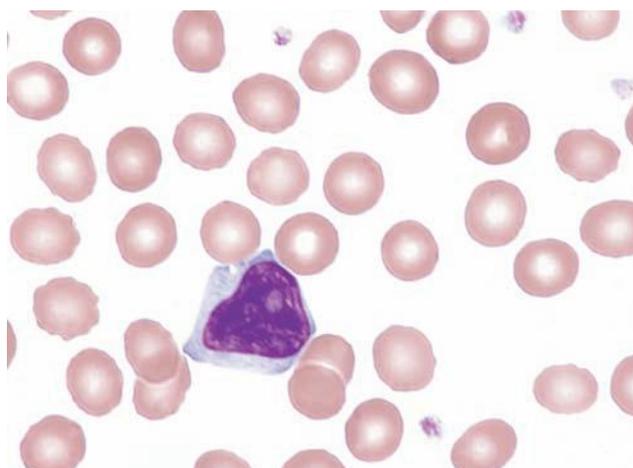


Figure 45-4 Peripheral blood film from a healthy newborn showing a benign lymphocyte with visible nucleoli ($\times 1000$).

Monocytes

The mean monocyte count of neonates is higher than adult values. At birth the average proportion of monocytes is 6%. During infancy and childhood, an average of 5% is maintained, except in the second and third weeks, when the proportion increases to around 9%. The count reaches adult levels at 3 to 5 months.²⁵

Neonatal Hematologic Response to Infection. The immune response of newborns is considered “immature,” with decreased responsiveness to agonists. This distinct immune response is postulated to be related to the demands of the fetal environment and the need to avoid response to maternal antigens.⁷¹ Sepsis in neonates is a common cause of morbidity, particularly in premature and low-birth-weight infants.⁷²⁻⁷⁶ Defective B cell response against polysaccharide agents, as well as abnormal cytokine release by neutrophils and monocytes, has been implicated.⁷⁷⁻⁸⁰ Because of the transient neutrophilia that occurs during the first 24 hours after birth, followed by a rapid decline, the neutrophil count is not a satisfactory index

of infection in the newborn.⁸¹ Newborns with bacterial infections frequently have neutrophil counts within or below the reference interval with a shift to the left. Thus many practitioners depend on the band count and its derived immature-to-total neutrophil ratio as an indicator of sepsis in neonates,⁸² although CD64 index, C-reactive protein, and procalcitonin levels have been suggested as more sensitive markers of sepsis in infants and children.^{82,83}

Platelet Values in the Newborn

The platelet count usually ranges from 150 to $400 \times 10^9/L$ for full-term and preterm infants, comparable to adult values (Table 45-4).^{84,85} Thrombocytopenia of fewer than 100×10^9 platelets/L may be seen in high-risk infants with sepsis or respiratory distress and neonates with trisomy syndromes, and investigation should be undertaken for underlying pathology.^{86,87} Platelets of a newborn infant show great variation in size and shape. Neonatal thrombocytopenia is discussed in Chapter 40.

Neonatal Hemostasis

The physiology of the hemostatic system in infants and children is different from that in adults (Chapter 37). The vitamin K-dependent coagulation factors (factors II, VII, IX, and X) are at about 30% of adult values at birth; they reach adult values after 2 to 6 months, although the mean values remain lower in children than in adults. Levels of factor XI, factor XII, prekallikrein, and high-molecular-weight kininogen are between 35% and 55% of adult values at birth, reaching adult values after 4 to 6 months. In contrast, the levels of fibrinogen, factor VIII, and von Willebrand factor are similar to adult values throughout childhood.^{88,89} Factor V decreases during childhood, with lower levels during the teen years as compared with adults. The physiologic anticoagulants and inhibitors of coagulation (protein C, protein S, and antithrombin) and a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13 (ADAMTS-13) that cleaves ultra-long von Willebrand factor multimers are reduced to about 30% to 40% at birth. Antithrombin reaches adult values by 3 months, whereas protein C does not reach adult levels until after 6 months.⁸⁸ In the fibrinolytic system, levels of plasminogen and α_2 -antiplasmin are similar to adult levels at birth, whereas levels of tissue plasminogen activator are low and levels of plasminogen activator inhibitor 1 (PAI-1) are increased (Table 45-5).⁸⁹ The hemostatic components are not

only changing in concentration over the first few weeks to months of life, but their values are also dependent on the gestational age of the child, and premature infants have different values at birth than term infants (Table 45-6).

Bleeding and Thrombosis

The reference intervals for the prothrombin time and partial thromboplastin time for neonates extend higher than those for adults; however, these values reach adult reference intervals by 6 months (Table 45-5). The risk of bleeding is not increased in a healthy newborn despite the decreased levels of the vitamin K-dependent coagulation factors, which is primarily related to the reduced levels of the physiologic anticoagulants protein C and protein S.⁹⁰

The risk of thrombosis is considerably less in neonates and children than in adults. However, two age-related peaks in frequency occur, the first in the neonatal period and the second in postpuberty adolescence.⁹¹ Central venous catheters, cancer, and chemotherapy are the most common risk factors in both of these groups.⁹²⁻⁹⁵ Hemorrhagic and thrombotic disorders are discussed in Chapters 38 and 39, respectively.

GERIATRIC HEMATOLOGY AND HEMOSTASIS

In 2010, there were 40 million people in the United States aged 65 and over, accounting for 13% of the population. The older population in 2030 is projected to be twice as large as in 2000, growing from 35 million to 75 million and representing 20% of the total U.S. population.⁹⁶ The life expectancy and quality of life of the elderly have improved dramatically in recent decades. Americans are living longer than ever before. Life expectancies at both age 65 and age 85 have increased. Under current conditions, people who survive to age 65 can expect to live an average of 19.2 more years—almost 5 years longer than people aged 65 in 1960. In 2009, the life expectancy of people who survived to age 85 was 7 years for women and 5.9 years for men.⁹⁶

Disease and disabilities are not a function of age, although age is a risk factor for many diseases. However, with the increase in the aging population, the incidence of age-related health conditions also is likely to increase. Thus the care of the elderly has become a growing concern as the life expectancy of the population continues to increase.

The elderly can be roughly divided into three age categories: the “young-old,” aged 65 to 74; the “old-old,” aged 74 to 84; and the “very old,” aged 85 and older.⁹⁷ The 85 and older age group is the fastest-growing segment of the elderly population.

Geriatric medicine is a rapidly growing branch of medicine. The use of inappropriate reference intervals may lead to unnecessary testing and investigations or, more importantly, result in failure to detect a critical underlying disease. The growing concern about the interpretation of hematologic data in reference to age is due partly to the tremendous heterogeneity in the aging process and partly to the difficulty in separating the effects of age from the effects of occult diseases that accompany aging.⁹⁸

TABLE 45-4 Platelet Count Reference Intervals for Full-Term and Preterm Infants

Age	Platelet Count ($\times 10^9/L$; mean \pm 1 SD)
Preterm infants, 27–31 wk	275 \pm 60
Preterm infants, 32–36 wk	290 \pm 70
Term infants	310 \pm 68
Healthy child/adult	300 \pm 50

Adapted from Oski FA, Naiman JL: Normal blood values in the newborn period. In *Hematologic problems in the newborn*, Philadelphia, 1982, WB Saunders. SD, Standard deviation.

TABLE 45-5 Reference Intervals for Coagulation Tests in the Healthy Full-Term Infant During the First 6 Months of Life

	Day 1	Day 5	Day 30	Day 90	Day 180	Adult
Screening Tests						
PT (Sec)	10.1–15.9	10.0–15.3	10.0–14.3	10.0–14.2	10.7–13.9	10.8–13.9
PT (INR)	0.53–1.62	0.53–1.48	0.53–1.26	0.53–1.26	0.61–1.17	0.64–1.17
PTT (Sec)	31.3–54.3	25.4–59.8	32.0–55.2	29.0–50.1	28.1–42.9	26.6–40.3
TCT (Sec)	19.0–28.3	18.0–29.2	19.4–29.2	20.5–29.7	19.8–31.2	19.7–30.3
Factor Assays						
Fibrinogen (mg/dL)	167–399	162–462	162–378	150–379	150–387	156–400
II (%)	26–70	33–93	34–102	45–105	60–116	70–146
V (%)	34–108	45–145	62–134	48–132	55–127	62–150
VII (%)	28–104	35–143	42–138	39–143	47–127	67–143
VIII (%)	50–178	50–154	50–157	50–125	50–109	50–149
VWF (%)	50–287	50–254	50–246	50–206	50–197	50–158
IX (%)	15–91	15–91	21–81	21–113	36–136	55–163
X (%)	12–68	19–79	31–87	35–107	38–118	70–152
XI (%)	10–66	23–87	27–79	41–97	49–134	67–127
XII (%)	13–93	11–83	17–81	25–109	39–115	52–164
PK (Fletcher, %)	18–69	20–76	23–91	41–105	56–116	62–162
HMWK (Fitzgerald, %)	6–102	16–132	33–121	30–146	36–128	50–136
XIIIa (%)	27–131	44–144	39–147	36–172	46–162	55–155
XIIIb (%)	30–122	32–180	39–173	48–184	20–170	57–137
Control Proteins						
Antithrombin (%)	39–87	41–93	48–108	73–121	84–124	79–131
α_2 -Macroglobulin (%)	95–183	98–198	106–194	126–226	149–233	52–120
C ₁ Inhibitor (%)	36–108	60–120	47–131	71–159	89–193	71–131
α_1 -Antitrypsin (%)	49–137	49–129	36–88	42–102	47–107	55–131
Heparin Cofactor II (%)	10–93	0–96	10–87	10–146	50–190	66–126
Protein C (%)	17–53	20–64	21–65	28–80	37–81	64–128
Protein S (%)	12–60	22–78	33–93	54–118	55–119	60–124
Fibrinolytic Proteins						
Plasminogen (%)	125–265	141–293	126–270	174–322	221–381	248–424
Tissue Plasminogen Activator (ng/mL)	5.0–18.9	4.0–10.0	1.0–6.0	1.0–5.0	1.0–6.0	1.4–8.4
α_2 -Antiplasmin (%)	55–115	70–130	76–124	76–140	83–139	68–136
Plasminogen Activator Inhibitor 1 (%)	20–151	0–81	0–88	10–153	60–130	0–110

From Andrew M, Paes B, Johnston M: Development of the hemostatic system in the neonate and young infant, *Am J Pediatr Hematol Oncol* 12(1):95-104, 1990.

HMWK, High-molecular-weight kininogen; INR, international normalized ratio; PK, prekallikrein; PT, prothrombin time; PTT, partial thromboplastin time; TCT, thrombin clotting time; VWF, von Willebrand factor.

This section focuses on hematologic changes in the elderly and discusses hematologic reference intervals for various geriatric age groups as well as hematopathologic conditions seen in the geriatric population.

Aging and Hematopoiesis

The aging process is associated with the functional decline of several organ systems, such as cardiovascular, renal, musculoskeletal, pulmonary, and bone marrow reserve. Certain cells lose their ability to divide (e.g., nervous tissue, muscles), whereas others, such as bone marrow and the gastrointestinal mucosa, remain mitotic. Marrow cellularity begins at 80% to 100% in infancy and decreases to about 50% after 30 years, followed by

a decline to 30% after age 65.⁹⁹⁻¹⁰¹ These changes may be due to a reduction in the volume of cancellous (trabecular or spongy) bone, along with an increase in fat, rather than to a decrease in hematopoietic tissue.¹⁰¹ Telomere shortening, which determines the number of divisions a cell undergoes, has not been definitively correlated to age-related hematopoietic stem cell exhaustion in humans¹⁰²; however, it is speculated to be associated with hematopoietic stem cell differentiation.¹⁰³

Assessment of Hematologic Parameters in Healthy Elderly Adults

In 1930, Wintrobe published hematologic reference intervals that are still in use today. These were derived from healthy

TABLE 45-6 Reference Intervals for Coagulation Tests in the Healthy Premature Infant of 30 to 36 Weeks' Gestation During the First 6 Months of Life

	Day 1	Day 5	Day 30	Day 90	Day 180	Adult
Screening Tests						
PT (Sec)	10.6–16.2	10.0–15.3	10.0–13.6	10.0–14.6	10.0–15.0	10.8–13.9
PT (INR)	0.61–1.70	0.53–1.48	0.53–1.11	0.53–1.32	0.50–1.48	0.64–1.17
PTT (Sec)	27.5–79.4	26.9–74.1	26.9–62.5	28.3–50.7	27.2–53.3	26.6–40.3
TCT (Sec)	19.2–30.4	18.8–29.4	18.8–29.9	19.4–30.8	18.9–31.5	19.7–30.3
Factor Assays						
Fibrinogen (mg/dL)	150–373	160–418	150–414	150–352	150–360	156–400
II (%)	20–77	29–85	36–95	30–106	51–123	70–146
V (%)	41–144	46–154	48–156	59–139	58–146	62–150
VII (%)	21–113	30–138	21–145	31–143	47–151	67–143
VIII (%)	50–213	53–205	50–199	58–188	50–187	50–149
VWF (%)	78–210	72–219	66–216	75–184	54–158	50–158
IX (%)	19–65	14–74	13–80	25–93	50–120	55–163
X (%)	11–71	19–83	20–92	35–99	35–119	70–152
XI (%)	8–52	13–69	15–71	25–93	46–110	67–127
XII (%)	10–66	9–69	11–75	15–107	22–142	52–164
PK (Fletcher, %)	9–57	25–75	31–87	37–121	40–116	62–162
HMWK (Fitzgerald, %)	9–89	24–100	16–112	32–124	41–125	50–136
XIIIa (%)	32–108	57–145	39–147	71–155	65–161	55–155
XIIIb (%)	35–127	68–158	57–157	75–167	67–163	57–137

From Andrew M, Paes B, Milner R, et al: Development of the human coagulation system in the healthy premature infant, *Blood* 72(5):1651-1657, 1988.

HMWK, High-molecular-weight kininogen; INR, international normalized ratio; PK, prekallikrein; PT, prothrombin time; PTT, partial thromboplastin time; TCT, thrombin clotting time; VWF, von Willebrand factor.

young adults, mostly medical students and nurses. What constitutes “normal” for elderly patients is a matter of considerable debate. There is controversy concerning the assignment of geriatric age-specific reference intervals, especially because aging is often accompanied by physiologic changes, and the prevalence of disease increases markedly. The baseline values for the elderly are the same reference intervals used for healthy adults. Proper interpretation of hematologic data, however, requires a complete understanding of the association between disease and older age. The next section highlights hematologic reference intervals for healthy elderly individuals with and without evidence of underlying disease.

Red Blood Cells

Most RBC parameters (e.g., RBC count, indices, and RDW) for healthy elderly do not show significant deviations from those for younger adults. There is a gradual decline in hemoglobin starting at middle age, with the mean level decreasing by about 1 g/dL during the sixth through eighth decades.¹⁰⁴ Men older than 60 years have average hemoglobin levels of 12.4 to 15.3 g/dL, whereas men aged 96 to 106 years have a mean hemoglobin level of 12.4 g/dL.^{105,106} The hemoglobin levels in women may increase slightly with age or remain unchanged. Elderly women have hemoglobin concentrations ranging from 11.7 to 13.8 g/dL. Males characteristically have higher hemoglobin levels than females, owing to the stimulating effect of androgens on erythropoiesis; however, the difference narrows as androgen levels decrease in elderly men and estrogen levels

decrease in older women.¹⁰⁷ Characteristically, the lowest hemoglobin levels are found in the oldest patients (Table 45-7).

Leukocytes

In the absence of any underlying pathologic condition, there are no statistically significant differences between the total leukocyte count and WBC differential for the young-old and old-old and those for middle-aged adults.¹⁰⁶ Some investigators, however, have reported a lower leukocyte count—3.1 to $8.5 \times 10^9/L$ —in individuals older than age 65, owing primarily to a decrease in the lymphocyte count. Others have reported a decrease in the lymphocyte and the neutrophil counts in women, but not in men, older than age 50.¹⁰⁷

Immune Response in the Elderly. Infectious diseases are an important cause of morbidity and mortality in the elderly. Aged adults are more susceptible to infection, take longer to recover from infection, and are often less responsive to vaccination.¹⁰⁸ The adverse changes that occur in the function of the immune system with age are called *immunosenescence*.¹⁰⁹ Although all components of immunity are affected, T cells appear to be the most susceptible.¹¹⁰ The thymus disappears by early middle age, and adults must then depend on their T-lymphocyte pool in the secondary tissues to mediate T cell-dependent immune responses.^{111,112} The number of naive T cells decreases in the elderly, which increases the dependence on memory T cells. T cells of the elderly have impaired responsiveness to mitogens and antigens as a result of decreased

TABLE 45-7 Hematologic Values in Ambulatory Healthy Adults*

	84–98 yr	30–50 yr
Red Blood Cells ($\times 10^{12}/L$)		
Males	4.8 \pm 0.4	5.1 \pm 0.2
Females	4.5 \pm 0.3	4.6 \pm 0.3
Hemoglobin (g/dL)		
Males	14.8 \pm 1.1	15.6 \pm 0.7
Females	13.6 \pm 1.0	14.0 \pm 0.8
Hematocrit (%)		
Males	43.8 \pm 3.3	45.3 \pm 2.2
Females	40.7 \pm 2.9	41.6 \pm 2.3
Mean Cell Volume (fL)		
Males	91.3 \pm 5.4	87.8 \pm 2.8
Females	90.5 \pm 4.1	90.5 \pm 5.0
Mean Cell Hemoglobin (pg)		
Males	31.0 \pm 2.0	30.1 \pm 1.6
Females	30.2 \pm 1.2	30.3 \pm 2.1
Mean Cell Hemoglobin Concentration (g/dL)		
Males	33.7 \pm 1.5	34.2 \pm 1.5
Females	33.4 \pm 1.2	33.5 \pm 1.5
White Blood Cells ($\times 10^9/L$)		
	7.6 \pm 0.5	8.8 \pm 0.4
Platelets ($\times 10^9/L$)		
	277 \pm 21	361 \pm 38
Neutrophils ($\times 10^9/L$)		
	4.5 \pm 0.3	5.9 \pm 0.3
Lymphocytes ($\times 10^9/L$)		
	1.9 \pm 0.3	1.9 \pm 0.8

Adapted from Zauber NP, Zauber AG: Hematologic data of healthy very old people, *JAMA* 357:2181-2184, 1987; and Chatta GS, Lipschitz DA: Aging of the hematopoietic system. In Hazzard WR, Blass JP, Halter JB, et al: *Principles of geriatric medicine and gerontology*, ed 5, New York, 2003, McGraw-Hill, p 767.

*Mean values \pm 1 standard deviation.

expression of costimulator CD28. There is also an alteration of T cell signaling with aging.¹¹³⁻¹¹⁵ B-lymphocyte function depends on T cell interaction. Thus the decreased ability to generate antibody responses, especially to primary antigens, may be the result of T cell changes rather than intrinsic defects in B lymphocytes.^{111,112,116,117}

Many neutrophil functions are decreased in the elderly, including chemotaxis, phagocytosis of microorganisms, and generation of superoxide. Studies indicate that these defects may be associated with changes to the cell membrane and/or to receptor signaling.¹⁰⁸

Monocytes and Macrophages

The aging process does not significantly affect the number of monocytes. Information on the effects of aging on monocyte and

macrophage function is limited and often conflicting.¹⁰⁸ Recent studies provide evidence for defects in the toll-like receptor (TLR) function in monocytes and macrophages in older individuals.¹⁰⁸ The TLRs play a crucial role in the immune response.¹¹⁸

Platelets

The platelet count does not significantly change with age. There have been reports of increased levels of β -thromboglobulin and platelet factor 4 in the α -granules and increased platelet phospholipid content.^{119,120} Thrombocytopenia may be drug induced or secondary to marrow infiltration of metastatic cancer, lymphoma, or leukemia (Chapter 40). Thrombocytosis can be categorized into primary and secondary (Chapter 40). Essential thrombocythemia is a myeloproliferative neoplasm characterized by sustained proliferation of megakaryocytes, resulting in platelet counts of $450 \times 10^9/L$ or greater (Chapter 33).¹²¹ Primary thrombocytosis can also be seen in chronic myelogenous leukemia. Secondary thrombocytosis, or reactive thrombocytosis, is associated with infections, rheumatoid arthritis, chronic inflammatory bowel disease, iron deficiency anemia, sickle cell anemia, and splenectomy.¹²²⁻¹²⁴

Anemia and the Elderly

Anemia is common in the elderly and its prevalence increases with age; however, anemia should not be viewed as an inevitable consequence of aging.^{125,126} Although anemia in the elderly is typically mild, it has been associated with substantial morbidity and mortality.^{127,128} WHO defines anemia as hemoglobin less than 13 g/dL in males and less than 12 g/dL in females. Using the WHO definition of anemia, the Third National Health and Nutrition Examination Survey (NHANES III), a national study that samples clinical specimens, found the prevalence of anemia in the United States in individuals over the age of 65 to be 11% in men and 10.2% in women. This proportion doubles in those aged 85 and above.¹²⁶ However, studies of the prevalence of anemia in the elderly show great variability in the definitions of anemia used, as well as differences in sample sizes, patient populations studied, countries in which the study was conducted, and study design. Thus estimates of the prevalence of anemia vary from 2.9% to 61% in elderly men and from 3.3% to 41% in elderly women.¹²⁹

The factors contributing to anemia include a decrease in bone marrow function, a decline in physical activity, nutritional deficiencies, cardiovascular disease, and chronic inflammatory disorders. Unexplained anemia, anemia due to hematologic malignancies, iron deficiency anemia, anemia related to therapy for nonhematologic malignancies, and anemia of chronic inflammation are the most common causes of anemia in the elderly.¹³⁰

Ineffective erythropoiesis and hypoproliferation are also seen in the elderly. Ineffective erythropoiesis is associated with vitamin B₁₂ or folate deficiency, myelodysplastic syndrome, sideroblastic anemia, and thalassemia. Hypoproliferative anemia often occurs secondary to iron deficiency, vitamin B₁₂ or folate deficiency, renal failure, hypothyroidism, chronic inflammation, or endocrine disease.^{130,131} To a lesser extent, the elderly are prone to anemias such as aplastic anemia,

hemolytic anemia, myelophthisic anemia, and anemia due to protein-calorie malnutrition.

The initial laboratory evaluation of anemia should include a complete blood count (CBC), a reticulocyte count, peripheral blood film review, and chemistry panel, along with other diagnostic tools, including iron studies (with ferritin), vitamin B₁₂, and folate levels. Table 45-8 indicates the types of anemia suggested by MCV and RDW results. In addition, assessment for signs of gastrointestinal blood loss, hemolysis, nutritional deficiencies, malignancy, chronic infection, renal or hepatic disease, or other chronic disease can provide important information for the evaluation of anemia in the elderly.

Anemia of Chronic Inflammation

Anemia of chronic inflammation, also known as *anemia of chronic disease*, frequently occurs with inflammatory disorders (e.g., rheumatoid arthritis, renal failure, liver disease), chronic infections, bedsores, collagen vascular disease, protein malnutrition, endocrine disorders, vitamin C deficiency, and neoplastic disorders.¹³¹⁻¹³⁹ This hypoproliferative anemia is the most common form of anemia in the hospitalized geriatric population.¹⁴⁰ The severity of the anemia generally correlates with the severity of the underlying disease.¹⁴¹ Hcpidin-induced inhibition of iron absorption in the intestines and iron mobilization from macrophages and hepatocytes, and impaired erythropoietin-dependent erythropoiesis triggered by inflammatory cytokines is involved in the pathogenesis of anemia of chronic inflammation (Chapter 20).^{142,143}

Iron Deficiency Anemia

Iron deficiency anemia is common in the elderly. Iron deficiency affects not only erythrocytes but also the metabolic pathways of iron-dependent tissue enzymes.¹³⁶ Hemoglobin synthesis is reduced, and even a minimal decrease can cause

profound functional disabilities in an elderly patient. The serum iron level decreases progressively with each decade of life, particularly in females. Nevertheless, healthy elderly adults usually have serum iron levels within the adult reference interval.

Iron deficiency anemia in the elderly is rarely due to dietary deficiency in industrialized nations because of the prevalence of iron fortification of grains, as well as a diet that includes meats containing heme iron. Iron deficiency in the elderly most frequently results from conditions leading to chronic gastrointestinal blood loss, including long-term use of nonsteroidal anti-inflammatory medications, gastritis, peptic ulcer disease, gastroesophageal reflux disease with esophagitis, colon cancer, and angiodysplasia.¹⁴⁴⁻¹⁴⁶ It also may be due to poor diet in an elderly individual who has lost the taste or desire for food or is unable to prepare nutritious meals. Chapter 20 discusses iron disorders in detail.

Ineffective Erythropoiesis

Ineffective erythropoiesis has been attributed not only to maturation disorders such as vitamin B₁₂ and folic acid deficiency but also to sideroblastic anemia, thalassemia, and myelodysplastic syndrome. Sideroblastic anemias are characterized by impaired heme synthesis, and abnormal globin synthesis occurs in the thalassemias (Chapters 20 and 28). Megaloblastic anemia results from defective synthesis of deoxyribonucleic acid (DNA) (Chapter 21) with compromised cell division but normal cytoplasmic development (i.e., asynchrony).¹⁴⁷⁻¹⁴⁹ These megaloblastic cells are more prone to destruction in the bone marrow, which results in ineffective erythropoiesis. Two causes of megaloblastic anemia are vitamin B₁₂ deficiency and folate deficiency. Myelodysplastic syndrome results in ineffective hematopoiesis due to mutations in hematopoietic stem cells and progenitor cells. It is more common in the elderly and is discussed below.

Vitamin B₁₂ Deficiency. Vitamin B₁₂ (cobalamin) deficiency causes a megaloblastic disorder in 5% to 10% of the elderly.¹³¹ It may be difficult to detect because anemia is present in only about 60% of patients.¹⁵⁰ Neurologic complications are found in 75% to 90% of individuals with clinically apparent vitamin B₁₂ deficiency.^{151,152} In the absence of anemia, neurologic symptoms may be the only indication. Even when anemia is present, it does not always manifest with the classic macrocytic and megaloblastic picture but may be normocytic.

Vitamin B₁₂ deficiency in the elderly has been attributed to inadequate intestinal absorption of food-bound vitamin B₁₂ rather than pernicious anemia or inadequate intake.^{143,153} Many elderly individuals have atrophic gastritis resulting in decreased gastric production of acid. In this condition there is low vitamin B₁₂ absorption because protein-bound vitamin B₁₂ is not dissociated from food proteins and therefore cannot bind to intrinsic factor for absorption. In addition, the loss of gastric acid can result in bacterial overgrowth, particularly with *Helicobacter pylori*, which also interferes with vitamin B₁₂ absorption.^{154,155} Inadequate vitamin B₁₂ absorption in the

TABLE 45-8 Classification of Geriatric Anemia Based on Typical Mean Cell Volume (MCV) and Red Cell Distribution Width (RDW)

MCV	RDW	Anemia
Normal	Normal	Anemia of chronic inflammation (some) Hemorrhagic anemia Leukemia-associated anemia
	High	Early iron deficiency anemia Mixed deficiency anemia (e.g., vitamin B ₁₂ and iron) Sideroblastic anemia
Low	Normal	Anemia of chronic inflammation (some)
	High	Iron deficiency anemia
High	Normal	Anemia associated with myelodysplastic syndrome
	High	Vitamin B ₁₂ deficiency anemia Folate deficiency anemia Hemolytic anemia

Note that this classification is not absolute because there can be an overlap of RDW values among some of the conditions in each MCV category.

elderly has also been reported in other infrequent conditions such as small bowel disorder, gastric resection, pancreatic insufficiency, resection of the terminal ileum, blind loop syndrome, and tropical sprue.¹⁵⁶ Pernicious anemia develops slowly and insidiously in patients when autoimmune antibodies to intrinsic factor or to parietal cells destroy their parietal cells so that they are left without intrinsic factor.¹⁵⁷ Given the high risk of vitamin B₁₂ deficiency in the aged and the risks of the condition, some authors have proposed that all elderly adults be screened periodically for vitamin B₁₂ deficiency.^{158,159} Chapter 21 discusses megaloblastic anemias.

Folate Deficiency. A second megaloblastic anemia that may be seen in the elderly results from folate deficiency. In contrast to vitamin B₁₂ deficiency, folic acid deficiency usually develops from inadequate dietary intake because the body stores little folate.¹⁶⁰ However, the incidence of low serum and RBC folate levels has declined in all age groups, including the elderly, since countries began to fortify their grains with folic acid in the 1990s.¹⁶¹ Alcoholic elderly patients are particularly prone to folic acid deficiency because alcohol interferes with folate absorption, enterohepatic recycling, metabolism, breakdown, and excretion (Chapter 21).^{162,163}

Hemolytic Anemia

Hemolytic anemias are characterized by a shortened RBC survival time. The three major types of hemolytic anemias are those caused by immunologic mechanisms; those due to intrinsic defects, such as an RBC membrane defect, abnormal hemoglobins, or RBC enzyme defects; and those resulting from extrinsic factors, such as mechanical or lytic processes.^{132,164} The elderly are at risk for drug-induced hemolytic anemia because they may take multiple medications. Drug-induced hemolytic anemia has been associated with high doses of antibiotics (penicillin, chloramphenicol, cephalosporins), several nonsteroidal anti-inflammatory drugs, quinidine, phenacetin, and others. Hemolytic anemias in the elderly also can result from collagen vascular diseases, infections, and chronic lymphocytic leukemia. The hemolytic anemias are discussed in Chapters 23 to 28.

Hematologic Neoplasia in Older Individuals

Although hematologic malignancies may occur at any age, certain disorders are common in those older than 50 years. A brief overview of these disorders is included in this chapter, with references to more detailed discussions.

Chronic Myeloid Neoplasms

The 2008 WHO classification of adult chronic myeloid neoplasms includes four broad categories: myelodysplastic syndrome; myeloproliferative neoplasms; myelodysplastic/myeloproliferative neoplasms; and myeloid and lymphoid neoplasms associated with eosinophilia and abnormalities of platelet-derived growth factor receptor α , platelet-derived growth factor receptor β , or fibroblast growth factor receptor 1. The chronic myeloid neoplasms show increased incidence in the elderly.

Myelodysplastic Syndrome. Myelodysplastic syndrome represents a heterogeneous group of clonal bone marrow disorders that may affect multiple cell lineages. Myelodysplastic syndrome is the most common hematologic malignancy in the elderly.^{130,165} The incidence of myelodysplastic syndrome increases from 6.6 cases per 100,000 for individuals aged 60 to 64 to 20.9 per 100,000 for those aged 70 to 74, and is 51.2 per 100,000 for those age 85 and older.¹⁶⁶ Typical features include progressive cytopenias, dyspoiesis in one or more cell lines, and an increase in blasts in the peripheral blood and bone marrow. Chapter 34 provides a complete discussion of myelodysplastic syndrome.

Myeloproliferative Neoplasms. Myeloproliferative neoplasms are monoclonal proliferations of hematopoietic stem cells with overaccumulation of RBCs, WBCs, or platelets in various combinations. Myeloproliferative disorders include chronic myelogenous leukemia, polycythemia vera, essential thrombocythemia, primary myelofibrosis, chronic eosinophilic leukemia not otherwise classified, mastocytosis, chronic neutrophilic leukemia, and unclassifiable myeloproliferative neoplasms. The incidence of myeloproliferative neoplasms increases from 6.1 per 100,000 for individuals aged 60 to 64, to 11.5 per 100,000 for those aged 70 to 74, and to 15.8 per 100,000 for those 85 years of age and older.¹⁶⁶ Chapter 33 discusses the myeloproliferative neoplasms.

Leukemia

Leukemia is a neoplastic disease characterized by a malignant proliferation of hematopoietic stem cells in the bone marrow, peripheral blood, and often other organs. Leukemia is broadly classified on the basis of the cell type involved (lymphoid or myeloid) and the stage of maturity of the leukemic cells (acute or chronic). Although the overall incidence of leukemia has decreased in the past 5 decades, there has been a disproportionately greater incidence of leukemia in the elderly (Table 45-9). The peaks in leukemia incidence seem to occur in the very young (age 1 to 4 years) and the very old (age 70 or older,

TABLE 45-9 SEER Incidence of Leukemia in the Elderly in the United States per 100,000 (2000–2010)*

Age at Diagnosis	All Leukemias	ALL	CLL	AML	CML	AMoL
65–69 yr	36.8	1.3	16.7	10.4	4.4	0.7
70–74 yr	49.4	1.4	21.9	14.9	5.7	0.9
75–79 yr	65.7	1.5	28.5	19.7	7.9	1.2
80–84 yr	77.8	1.6	32.4	23.2	9.7	1.4
≥85 yr	88.5	1.8	37.5	22.7	10.1	1.5

Adapted from Howlader N, Noone AM, Krapcho M, et al, editors: SEER cancer statistics review, 1975-2010, Bethesda, Md, 2010, National Cancer Institute. Based on November 2012 SEER data submission, posted to the SEER website in 2013. Available at: http://seer.cancer.gov/csr/1975_2010/. Accessed July 10, 2013.

ALL, Acute lymphoblastic leukemia; AML, acute myeloid leukemia; AMoL, acute monocytic leukemia; CLL, chronic lymphocytic leukemia; CML, chronic myelogenous leukemia.

*Surveillance, Epidemiology, and End Results (SEER) Incidence Source: SEER 18 areas.

especially men). Acute myelogenous leukemia (Chapter 35) and chronic lymphocytic leukemia (Chapter 36) show the most dramatic age-related increase in incidence.¹⁶⁶

Geriatric Hemostasis

Age-related changes occur in the vascular and hemostatic systems, including alterations in platelets, coagulation, and fibrinolytic factors. These changes are thought to contribute to the increased incidence of thrombosis in the elderly. The rate of venous thromboembolism, for example, increases from 1 per 10,000 in the young (25 to 30 years of age) to 8 per 1000 in the elderly (85 years and older).¹⁶⁷ Approximately 60% of venous thrombosis events occur in those aged 70 years and older (Chapter 39).¹⁶⁸

Fibrinogen, factor V, factor VII, factor VIII, factor IX, factor XIII, high-molecular-weight kininogen, and prekallikrein increase in healthy individuals as they age.¹⁶⁹ Fibrinogen, which has been implicated as a primary risk factor for thrombotic disorders, increases approximately 10 mg/dL per decade in the elderly (65 to 79 years),¹⁷⁰ from 280 mg/dL to over 300 mg/dL.

Elevated levels of factor VIII also have been associated with increased risk of venous thrombosis.¹⁷¹ Studies of the association between factor VII and venous thrombosis have yielded conflicting findings.¹⁶⁹

PAI-1, the major inhibitor of fibrinolysis, increases with aging. PAI-1 has been shown to promote age-dependent thrombosis in animal models and could play an important role in causing hypercoagulability in the elderly.¹⁷²⁻¹⁷⁴

Platelets increase in activity with age, as evidenced by a decrease in bleeding time as males age from 11 to 70 years¹⁷⁵ and an increase in markers of platelet activation, platelet β -thromboglobulin, and platelet factor 4.¹⁷⁶ Increased platelet activity with aging is also associated with increased platelet phospholipids, which suggests an age-related increase in platelet transmembrane signaling.¹⁷⁷

Many conventional risk factors associated with venous thrombosis are likely to also increase the risk of thrombosis in the elderly. These factors include immobility, malignant disease, comorbidities, and prescription drugs that influence coagulation or platelet function.^{143,167}

SUMMARY

- The newborn infant, preadolescent child, and elderly adult exhibit profound hematologic differences from one another.
- Newborn hematologic parameters continue to change and evolve over the first few days, weeks, and months of life. Laboratory results must be assessed in light of gestational age, birth weight, and developmental differences between newborns and older infants.
- The erythrocytes of newborn infants are markedly macrocytic at birth. A condition known as *physiologic anemia of infancy* occurs after the first few weeks of life. Infants born prematurely also experience a decrease in hemoglobin concentration, which is termed *physiologic anemia of prematurity*.
- Iron deficiency is the most frequent cause of anemia in children.
- Fluctuations in the number of WBCs are common at all ages but are greatest in infants. Leukocytosis is typical at birth for healthy full-term and preterm infants, with a mean of 22×10^9 cells/L (range, 9 to 30×10^9 cells/L) at 12 hours of life. There is an increase in segmented neutrophils, bands, and occasional metamyelocytes with no evidence of disease.
- Sepsis in neonates is a common cause of morbidity, particularly in premature and low-birth-weight infants. Defective B cell response against polysaccharide agents, as well as abnormal cytokine release by neutrophils and monocytes, have been implicated.
- Although hemostatic values are different in infants and children from those in adults, this population is not at increased risk of bleeding or thrombosis.
- There is a gradual decline in hemoglobin starting at middle age, and the mean level decreases by about 1 g/dL during the sixth through eighth decades.
- Although anemia is common in elderly patients, it is not a normal occurrence in the aging process. The cause of anemia may be multifactorial in elderly patients.
- Iron deficiency anemia, anemia of chronic inflammation, and megaloblastic anemia related to vitamin B₁₂ deficiency are the most common anemias seen in the elderly.
- *Immunosenescence* refers to the adverse changes in the immune system associated with aging.
- The elderly experience an increased frequency of many neoplastic and malignant disorders, such as acute and chronic leukemia, myelodysplastic syndromes, and myeloproliferative neoplasms.
- The elderly are at an increased risk of thrombosis associated with age-related changes in the vascular and hemostatic systems.

Now that you have completed this chapter, go back and read again the case study at the beginning and respond to the questions presented.

REVIEW QUESTIONS

Answers can be found in the Appendix.

- The CBC results for children (aged 3 to 12 years) differ from those of adults chiefly in what respect?
 - NRBCs are present.
 - Notable polychromasia is seen, indicating increased reticulocytosis.
 - Platelet count is lower.
 - The percentage of lymphocytes is higher.
- Physiologic anemia of infancy results from:
 - Iron deficiency caused by a milk-only diet during the early neonatal period
 - Increased oxygenation of blood and decreased erythropoietin
 - Replacement of active marrow with fat soon after birth
 - Hb F and its diminished oxygen delivery to tissues
- The CBC report on a 3-day-old neonate who was born 6 weeks prematurely shows a decrease in hemoglobin compared with the value obtained 2 days earlier. Which of the following should be considered as an explanation for this result when no apparent source of hemolysis or bleeding is evident?
 - The sample was collected from a vein at the time that an intravenous line was inserted.
 - The sample was collected by heel puncture rather than finger puncture because of the infant's small size.
 - The umbilical cord was clamped quickly to begin appropriate treatment for a preterm infant.
 - The infant has become dehydrated.
- Morphologically, the hematogones in newborns are:
 - Similar to those seen in megaloblastic anemia
 - Easily confused with leukemic blasts
 - Monocytoid in appearance
 - Similar to adult lymphocytes
- The most frequent cause of anemia in childhood is:
 - Vitamin B₁₂ deficiency
 - Drug-related hemolysis
 - Iron deficiency
 - Folate deficiency
- As age increases, the hemoglobin level of elderly adults:
 - Remains unchanged from that of middle-aged adults
 - Increases due to diminished respiration and poor tissue oxygenation
 - Decreases for reasons that are unclear
 - Becomes comparable to that of newborns
- Which of the following are the most common anemias in the elderly population?
 - Megaloblastic anemia and iron deficiency anemia
 - Sideroblastic anemia and megaloblastic anemia
 - Myelophthistic anemia and anemia of chronic inflammation
 - Iron deficiency anemia and anemia of chronic inflammation
- When iron deficiency is recognized in an elderly individual, the cause is usually:
 - An iron-deficient diet
 - Gastrointestinal bleeding
 - Diminished absorption
 - Impaired incorporation of iron into heme as a result of telomere loss
- Which of the following conditions is least likely in an elderly individual?
 - Acute lymphoblastic leukemia
 - Multiple myeloma
 - Myelodysplasia
 - Chronic lymphocytic leukemia
- The multiple medications used by the elderly makes this population more prone to:
 - Anemia of chronic inflammation
 - Megaloblastic anemia
 - Hemolytic anemia
 - Iron deficiency anemia

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Answers

CHAPTER 2

Case Study

1. This item describes two deficiencies. First, the hematology laboratory scientist should have washed his/her hands after removing the gloves and before leaving the laboratory. Second, the hematology laboratory scientist should have removed his/her laboratory coat before going to the meeting.
2. This item describes a deficiency. Storage of food in a specimen refrigerator is prohibited.
3. This may or may not be a deficiency. The laboratory employees may have had on a personal laboratory coat. A second laboratory coat could have been obtained by the employees to wear in public areas. Some laboratories require different colored lab coats for public areas.
4. No deficiency is indicated. Fire extinguishers should be placed every 75 feet.
5. This item describes a deficiency. Fire extinguishers should be inspected monthly and maintained annually.
6. This item represents a deficiency. All chemicals should be labeled.
7. This item represents a deficiency. The 1:10 bleach solution should be made fresh daily.
8. No deficiency is indicated. Gloves should be worn by all personnel handling specimens.
9. No deficiency is indicated. Safety data sheets can be received by fax.
10. This item describes a deficiency. Chemicals should not be stored alphabetically, but according to storage requirements specified in the safety data sheets.

Review Questions

1. d; 2. b; 3. c; 4. c; 5. a; 6. b; 7. c; 8. b; 9. b; 10. b; 11. d

CHAPTER 3

Case Studies

Case 1

The proper procedure is to ask the patient to state his/her full name and then confirm by asking his/her birth date and/or address. The phlebotomist should not prelabel tubes; tubes should be labeled after the blood is drawn and before leaving the patient.

Case 2

Test results that can be affected by this selection of tubes and order of draw include the prothrombin time (PT), potassium, and type and screen.

- The light blue stopper tube for the PT should not have been collected after the serum separator tube (which contains an inert gel and clot activator). The clot activator could contaminate the blue stopper tube, activate coagulation factors, and cause an error in the PT results.

- The green stopper tube for potassium should not have been collected after a lavender stopper tube (which contains EDTA, usually as a potassium salt). The potassium-EDTA could be carried over into the green stopper tube and falsely elevate the potassium level. The potassium could, however, be assayed in the serum separator tube.
- The type and screen cannot be done on blood from the serum separator tube because the gel interferes with blood bank procedures; the lavender stopper tube, however, could be used for the type and screen. Box 3-2 contains the correct order of draw for evacuated tubes.

Review Questions

1. a; 2. b; 3. b; 4. c; 5. b; 6. b; 7. b; 8. d; 9. b; 10. c; 11. c; 12. a

CHAPTER 4

Case Study

The following things should be checked:

Is the slide right side up? This is the most common cause of inability to focus a slide under oil when it has been focused under 10 \times and 40 \times objectives.

If the slide is right side up, continue by checking the following:

- Is there sufficient oil on the slide? If not, clean off all the residual oil first and then apply another drop of oil.
- Is the objective screwed in tightly? If not, tighten the objective.
- If the slide has a coverslip, is there more than one coverslip on the slide? If so, gently remove the top coverslip.
- Has oil seeped into the seal on the oil objective? Examine by removing the objective and use an inverted eyepiece as a magnifier to check the seal. If the seal is broken, the objective must be replaced.

Review Questions

1. b; 2. c; 3. a; 4. b; 5. c; 6. c; 7. d; 8. a; 9. c; 10. d; 11. b

CHAPTER 5

Case Study

1. This is a systematic error because the magnitude of error remains constant at three ranges of test results.
2. It is not acceptable to continue using the instrument or to simply subtract the systematic error from test sample results. All the samples in a two out-of-control test run must be re-assayed after the error is corrected.
3. Determine from the quality control charts at what moment the error occurred. Investigate potential changes in instrument settings, calibration, reagent changes, or instrument malfunction that may have occurred at the time the error was recorded.

Review Questions

1. d; 2. c; 3. b; 4. b; 5. b; 6. a; 7. d; 8. d; 9. c; 10. c; 11. c; 12. a; 13. b; 14. c; 15. a; 16. b

CHAPTER 6**Review Questions**

1. b; 2. b; 3. a; 4. b; 5. c; 6. a; 7. d; 8. d; 9. c; 10. b; 11. a; 12. d; 13. b; 14. a

CHAPTER 7**Review Questions**

1. a; 2. d; 3. c; 4. a; 5. b; 6. c; 7. c; 8. b; 9. a; 10. d; 11. a; 12. b

CHAPTER 8**Case Study**

- When the blood is not well oxygenated, the bone marrow responds by producing more red blood cells to carry more oxygen.
- The hormone that stimulates RBC production is erythropoietin (EPO). The peritubular cells of the kidney detect hypoxia. A hypoxia-sensitive transcription factor is produced that moves to the peritubular cell nucleus and upregulates transcription of the EPO gene. EPO acts by preventing apoptosis of the erythroid colony-forming unit. In RBC precursors, it also shortens the cell cycle time between mitoses and reduces the number of mitotic divisions; and it promotes early release of reticulocytes from the bone marrow.
- Once the patient was receiving oxygen therapy, hypoxia diminished and EPO production also declined. Thus, production of new RBCs slowed. At the same time, RBCs reaching 120 days of age were removed from the circulation. Thus the total number of circulating RBCs decreased.

Review Questions

1. c; 2. a; 3. d; 4. b; 5. b; 6. d; 7. a; 8. d; 9. a; 10. c; 11. b; 12. b

CHAPTER 9**Case Study**

- A reducing agent is able to donate an electron to an oxidized compound so that the oxidized compound has one fewer unpaired proton. The compound receiving the electron becomes reduced and the donating compound becomes oxidized.
- When heme iron is oxidized, the molecule cannot carry oxygen and patients become cyanotic. Because vitamin C eliminated the cyanosis, it must be able to reduce methemoglobin and restore the oxygen-carrying capacity of the blood.
- Because this condition affected brothers, a hereditary condition was suggested in which hemoglobin became oxidized more than is usual. (The condition affecting these brothers was later identified as a hereditary deficiency of methemoglobin reductase.)

Review Questions

1. a; 2. c; 3. b; 4. d; 5. b; 6. b; 7. a; 8. c; 9. a; 10. a; 11. a; 12. a

CHAPTER 10**Case Study**

- The mother's and infant's hemoglobin results were within the reference intervals. (Reference intervals: adult women, 12.0 to 15.0 g/dL; newborns, 16.5 to 21.5 g/dL.)

- The major hemoglobin at birth is Hb F. It has a high oxygen affinity because it weakly binds 2,3-BPG resulting in decreased delivery of oxygen to the tissues. The hypoxia triggers an increase in secretion of erythropoietin by the fetal kidney, which results in an increase in the production and release of red blood cells from the fetal bone marrow. The resultant increase in red blood cell count, hemoglobin concentration, and hematocrit compensates for the high Hb F oxygen affinity and reduced oxygen transfer to tissues. The Hb F concentration gradually decreases to adult physiologic levels by 1 to 2 years of age as most of the Hb F is replaced by Hb A.
- The hemoglobin assay measures concentration; high performance liquid chromatography (and hemoglobin electrophoresis) identifies and quantifies hemoglobin types.
- These are the expected results for hemoglobin fractions for a healthy mother and infant. In the second and third trimesters of fetal life, the α - and γ -globin genes are activated producing α and γ globin chains that combine to form Hb F. In late fetal life, γ - β switching begins in which transcription of the β -globin gene begins to be activated and the γ -globin gene begins to be repressed. With the activation of the β -globin gene, the β chains combine with the α chains to form Hb A. The Hb F level decreases from 60% to 90% at birth to 1% to 2% by 1 to 2 years of age, while the Hb A increases from 10% to 40% at birth to greater than 95% at 1 to 2 years of age and throughout life. The synthesis of Hb A₂ begins shortly before birth and remains at less than 3.5% throughout life.

Review Questions

1. d; 2. a; 3. a; 4. a; 5. a; 6. d; 7. c; 8. b; 9. d; 10. b; 11. c

CHAPTER 11**Case Study**

- Iron loss via blood donations and normal physiologic loss was not compensated by diet or supplementation.
- Adaptation to the low iron levels. Iron stores of ferritin were mobilized first. But when storage iron declined, hepcidin levels declined, and as a result, duodenal iron absorption increased.
- Ferritin
- Transferrin saturation reflects the proportion of transferrin binding sites for iron that are actually filled with iron during transit in the plasma. Transferrin level is an indirect indicator of the iron storage compartment while serum iron is the transport compartment, so transferrin saturation effectively reflects both compartments.

Review Questions

1. c; 2. b; 3. c; 4. c; 5. c; 6. b; 7. d; 8. b; 9. d; 10. c; 11. b; 12. d

CHAPTER 12**Case Study**

- The patient had an asthmatic attack. Eosinophils play an important role in the initiation and maintenance of symptoms. Eosinophils release basic proteins, lipid mediators, and reactive oxygen species that cause inflammation and damage to the mucosal cells lining the airway.
- Eosinophils are typically elevated in the peripheral blood and also in the sputum of asthmatic patients. The number of eosinophils in the blood correlates with the severity of the case.
- IL-5 plays an important role in the differentiation and proliferation of eosinophils. Monoclonal antibodies to IL-5 block eosinophil development. Since eosinophils are reduced, the symptoms of asthma are controlled.

Review Questions

1. b; 2. d; 3. a; 4. c; 5. b; 6. c; 7. c; 8. a; 9. b; 10. d

CHAPTER 13

Case Study

- Bleeding characterized by petechiae, purpura, and ecchymoses is known as *mucocutaneous bleeding*, also called *systemic bleeding*. By contrast, *anatomic bleeding* is bleeding into soft tissue, muscles, joints, or body cavities.
- Thrombocytopenia*, or low platelet count, is a common cause of mucocutaneous bleeding. Another is diseases that weaken vascular collagen such as scurvy.
- No, the bone marrow megakaryocyte estimate is high, indicating an increase in platelet production.
- Thrombopoietin* and *interleukin-11* have the greatest effect on recruitment and proliferation of megakaryocytes and their progenitors. Also involved in early progenitor recruitment are interleukin-3 and interleukin-6. Other cytokines and hormones that participate synergistically with thrombopoietin and the interleukins are KIT ligand, also called *stem cell factor* or *mast cell growth factor*; granulocyte-macrophage colony-stimulating factor; granulocyte colony-stimulating factor; and erythropoietin.

Review Questions

1. d; 2. d; 3. c; 4. b; 5. d; 6. d; 7. a; 8. a; 9. c; 10. d

CHAPTER 14

Case Studies

Case 1

- $HGB \times 3 = HCT \pm 3$
 $15 \times 3 = 45 \pm 3 (42-48)$
- Hemoglobin can be falsely elevated by lipemia, increased WBC count, or presence of Hb S or Hb C. Hematocrit can be falsely decreased by a short draw in an EDTA-anticoagulated tube causing RBC shrinkage, or contamination of the specimen with intravenous fluids. In the microhematocrit method, false decreases can be caused by improper sealing of the capillary tube, errors in reading the microhematocrit reader, excessive centrifugation, and improper mixing of the specimen.
- For lipemia, replace lipemic plasma with an equal amount of saline and retest; or use a plasma blank. For increased WBC count, centrifuge the hemoglobin/reagent solution and read the % T of the supernatant (manual procedure). For specimens with Hb S or Hb C, make a 1 : 2 dilution of blood with distilled water and multiply the result by 2. For the microhematocrit, check if the specimen tube was filled to the proper level, and ensure the procedure is performed correctly.

Case 2

- $MCV = 59 \text{ fL}$; $MCH = 18.1 \text{ pg}$; $MCHC = 30.7 \text{ g/dL}$.
- Microcytic, hypochromic red blood cells
- Examine the patient's peripheral blood film

Case 3

- The sodium concentration could affect the hematocrit. The sample electrolyte concentration is used to correct the measured conductivity prior to reporting hematocrit results. Factors that affect sodium concentration will therefore also affect the hematocrit.
- A high sodium concentration would falsely decrease the hematocrit.
- Factors that decrease the hematocrit by this method are low total protein, settling of red blood cells in the collection device, presence

of cold agglutinins, and specimen contamination by intravenous solutions.

Review Questions

1. b; 2. c; 3. c; 4. d; 5. d; 6. b; 7. c; 8. c; 9. a; 10. d

CHAPTER 15

Review Questions

1. d; 2. a; 3. d; 4. c; 5. c; 6. Impedance - c; RF - b; optical scatter - a; 7. b; 8. b; 9. c; 10. Abbott CELL-DYN Sapphire - b; Siemens ADVIA 2120i - c; Sysmex XN-1000 - d; Beckman Coulter UniCel DxH 800 - a.

CHAPTER 16

Case Study

- The patient's hemoglobin shows neither anemia or polycythemia; hence it is normal. Red blood cells are normocytic and normochromic with no anisocytosis. The blood picture shows leukocytosis and thrombocytopenia. The mean platelet volume is slightly low, which suggests small average platelet volume. There is no white blood cell (WBC) differential.
- The platelet count and WBC count should be questioned because of platelet clumping. EDTA-induced pseud thrombocytopenia and pseudoleukocytosis most likely occurred.
- The specimen should be redrawn in sodium citrate and processed through the automated analyzer. The new WBC and platelet counts should then be adjusted for the sodium citrate dilution by multiplying the results by the dilution factor 10/9 or 1.1. The following are the new results:
 - WBCs for specimen drawn in sodium citrate: $(8.4 \times 10^9/L) \times 1.1 = 9.2 \times 10^9/L$ (the corrected WBC count)
 - Platelets for specimen drawn in sodium citrate: $(231 \times 10^9/L) \times 1.1 = 254 \times 10^9/L$ (the corrected platelet count)

Review Questions

1. d; 2. c; 3. a; 4. c; 5. b; 6. c; 7. a; 8. b; 9. b; 10. a

CHAPTER 17

Case Study

- Bone marrow cellularity, estimated from the core biopsy specimen, or the aspirate if a biopsy specimen is unavailable, provides information on blood cell production.
- The ratio is 9:1, which indicates myeloid hyperplasia.
- When a bone marrow aspirate or core biopsy specimen is reviewed, the normal megakaryocyte distribution is 2 to 10 per low-power field. Counts outside these limits are characterized as decreased or increased megakaryocytes. Megakaryocyte morphology is also reviewed for diameter, granularity, and nuclear lobularity.

Review Questions

1. c; 2. b; 3. c; 4. a; 5. d; 6. c; 7. d; 8. b; 9. b; 10. b; 11. b

CHAPTER 18

Case Study

- Tube 3 or the least bloody tube.
- A 1:53 dilution with saline is necessary for a satisfactory cytocentrifuge slide.
- Bacteria.
- The most likely diagnosis is bacterial meningitis.

Review Questions

1. b; 2. a; 3. c; 4. b; 5. a; 6. b; 7. c; 8. d; 9. c; 10. a

CHAPTER 19

Case Study

- Anemia is not a disease or diagnosis in itself but is the symptom of an underlying disorder. A complete history and physical examination are necessary to help identify the cause(s) of the anemia. If the underlying cause is not determined and corrected, the patient will continue to be anemic. Questions regarding lifestyle, medications, and bleeding history are only some of the questions that should be asked.
- The reticulocyte count differentiates anemias into those involving impaired production (decreased reticulocyte count) and increased destruction (increased reticulocyte count). Anemia can also be classified on the basis of mean cell volume into normocytic, microcytic, or macrocytic. With that knowledge, appropriate laboratory testing can be ordered to determine the cause.
- The peripheral blood film yields valuable information about the volume and hemoglobin content of the erythrocytes as well as any abnormal shapes, which may be correlated with specific causes. Some anemias are also associated with white blood cell and/or platelet abnormalities, which may be noted on the blood film.

Review Questions

1. c; 2. b; 3. d; 4. c; 5. c; 6. b; 7. c; 8. d; 9. b; 10. c; 11. d

CHAPTER 20

Case Study

- The patient's results demonstrate a severe hypochromic, microcytic anemia with anisocytosis. There is no evidence of a bone marrow response as there is no polychromasia mentioned in the morphology which does note unspecified poikilocytosis, anisocytosis, hypochromia, and microcytosis, all consistent with the numerical values. The white blood cells are unremarkable in number, distribution, and morphology as are the platelets.
- Hypochromic, microcytic anemias to be considered include iron deficiency anemia, thalassemia, hemoglobin E disease, sideroblastic anemias, and possibly, anemia of chronic inflammation.
- Thalassemia and hemoglobin E disease can be eliminated because they are not conditions that would be acquired late in life.
- Anemia of chronic inflammation could be eliminated in this case because the woman is otherwise healthy. Although iron deficiency anemia is not as common in women after menopause, it is probably the most likely of the remaining possibilities for an anemia that is this severe.
- Iron studies, including ferritin, would be useful in clarifying the patient's diagnosis. Assuming that she is iron deficient, the ferritin, total serum iron, and percent saturation should all be decreased, whereas total iron-binding capacity (TIBC) would be expected to be increased. Upon hospitalization, the patient was immediately placed on oxygen while laboratory tests were ordered. With the confirmation by the hospital laboratory of a dangerously low hemoglobin, transfusions were ordered, and the patient received 3 units of packed cells over the first 2 days of hospitalization. The transfusions were administered very slowly so as not to stress her cardiovascular system with added volume. Noting the hypochromic, microcytic blood picture, the physician ordered iron studies on blood specimens drawn before the transfusions. The results

were as follows: serum iron decreased, TIBC increased, percent saturation decreased, and ferritin decreased. The possibility of gastrointestinal bleeding as a cause for iron deficiency was investigated. Results of tests for occult blood in the stool were negative. The hospital dietitian assessed the patient's usual diet of tea, toast, canned soup, and crackers and determined that it was quite inadequate not only in iron, but also in other important nutrients. The physician concluded that the patient's dietary iron deficiency had developed slowly, which had allowed her to adapt to the exceedingly low hemoglobin level. Furthermore, her low level of activity meant that she rarely experienced the effects of the anemia. She was started on a course of oral iron supplementation and arrangements were made for her to receive one balanced meal daily from the Meals on Wheels program sponsored through a community service organization for senior citizens. She was quite responsible about taking her iron supplements, and her hemoglobin was within the reference interval within 3 months.

Review Questions

1. b; 2. a; 3. d; 4. a; 5. c; 6. a; 7. d; 8. c; 9. b; 10. d; 11. b; 12. d

CHAPTER 21

Case Study

- The complete blood count findings for this patient (notably macrocytic, normochromic anemia; pancytopenia; hypersegmentation of neutrophils; and oval macrocytes) were consistent with the physician's suspicion of megaloblastic anemia as suggested by the clinical findings.
- Although the relative reticulocyte count was within the reference interval of 0.5% to 2.5%, and the calculated absolute reticulocyte count (approximately $40 \times 10^9/L$) was within the reference interval of 20 to $115 \times 10^9/L$, the calculated reticulocyte production index was 0.5, which was clearly inadequate to compensate for a substantial anemia (Chapter 14).
- The patient's vitamin assays point to a deficiency of vitamin B₁₂, substantiated by an increase in serum methylmalonic acid.
- Based on these results, a test for intrinsic factor blocking antibodies would be appropriate. However, the physician also inquired further about the patient's dietary habits and learned that he enjoyed dishes of raw fish obtained from the surrounding lakes. Therefore, the physician ordered a stool analysis for ova and parasites. The study indicated the presence in the stool of both eggs and proglottids of the fish tapeworm *Diphyllobothrium latum*. The patient was treated with a suitable purgative, and the scolex of the tapeworm was discovered in a stool sample after a single treatment. The patient was counseled on the proper preparation of fresh fish to avoid reinfection. He received injections of cyanocobalamin to replenish his vitamin B₁₂ stores. His hemoglobin returned to normal over the next month, and his neurologic symptoms subsided.

Review Questions

1. d; 2. c; 3. c; 4. b; 5. a; 6. b; 7. c; 8. d; 9. a; 10. c

CHAPTER 22

Case Study

- The term used to describe a decrease in all cell lines is *pancytopenia*.
- Acquired aplastic anemia should be considered due to the pancytopenia, reticulocytopenia, bone marrow hypocellularity, normal vitamin B₁₂ and folate levels, absence of blasts and abnormal cells

in the bone marrow and peripheral blood, normal myelopoiesis and megakaryopoiesis, and history of autoimmune hepatitis.

- An increase in blasts or reticulin in the bone marrow suggests a diagnosis of myelodysplasia or leukemia.
- The extent of the patient's bone marrow hypocellularity, her hemoglobin concentration, and neutrophil and platelet counts place her disorder in the severe aplastic anemia category.
- Because of her age and the severity of her aplastic anemia, hematopoietic stem cell transplant is the treatment of choice if she has an HLA-identical sibling. If an HLA-identical sibling is not available, an HLA-matched unrelated donor or immunosuppressive therapy (antithymocyte globulin and cyclosporine) may be considered. Blood product replacement should be given judiciously to avoid alloimmunization. In general, red blood cells would be transfused if the patient had symptoms of anemia, whereas platelet transfusions would be given if her platelet count fell below $10 \times 10^9/L$.

Review Questions

1. c; 2. d; 3. b; 4. d; 5. b; 6. d; 7. c; 8. d; 9. c; 10. d; 11. a

CHAPTER 23

Case Study

- Intravascular hemolysis is suspected in the patient because the color of the urine suggests oxidized hemoglobin.
- Tests for serum haptoglobin, serum unconjugated (indirect) bilirubin, serum lactate dehydrogenase, plasma hemoglobin, and urine hemoglobin and examination of a peripheral blood film can differentiate the mechanism of hemolysis as fragmentation or macrophage-mediated.
- Due to the likelihood that the patient had hemoglobinuria, fragmentation hemolysis was suspected. Therefore, the serum haptoglobin would be markedly decreased, while the serum lactate dehydrogenase and plasma hemoglobin levels would be increased, if measured. Routine urinalysis should yield positive results for blood on the test strip with no intact red blood cells in the urine sediment. The serum indirect bilirubin does not increase immediately after an episode of intravascular hemolysis, but should begin to increase within several days. The peripheral blood film may demonstrate schistocytes immediately, but reticulocytosis several days later.

Review Questions

1. a; 2. b; 3. d; 4. b; 5. c; 6. a; 7. d; 8. b; 9. c; 10. c

CHAPTER 24

Case Study

- On the basis of the patient's jaundice and splenomegaly, history of gallstones, family history of anemia, low hemoglobin, increased mean cell hemoglobin concentration and red cell distribution width, and spherocytes and polychromasia on the peripheral blood film, hereditary spherocytosis (HS) is suspected.
- Additional laboratory tests to confirm HS should demonstrate increased hemolysis (increased serum indirect bilirubin level and lactate dehydrogenase activity, decreased serum haptoglobin level), increased erythropoiesis to compensate for the premature hemolysis (increased reticulocyte count), and the nonimmune nature of the hemolysis (negative result on the direct antiglobulin test). Testing family members to establish a mode of inheritance is desirable. The

osmotic fragility test is expected to show increased fragility and the eosin-5'-maleimide (EMA) binding test is expected to show low mean fluorescence intensity of the red blood cells when measured in a flow cytometer. However, special tests are not required for diagnosis of HS in a patient with a familial inheritance pattern and the typical clinical and laboratory findings.

- HS is an inherited intrinsic hemolytic anemia caused by a mutation that disrupts the vertical protein interactions in the red blood cell (RBC) membrane. Various mutations in five known genes can result in the HS phenotype. The defective membrane protein causes the RBCs to lose unsupported lipid membrane over time due to a local disconnection between transmembrane proteins and the cytoskeleton. The loss of membrane with minimal loss of cell volume results in a decreased surface area-to-volume ratio and the formation of spherocytes. Spherocytes do not have the deformability of normal biconcave discoid RBCs. As the cells repeatedly go through the spleen, they lose more membrane due to splenic conditioning and eventually become trapped in the spleen and removed by the splenic macrophages. The RBC membrane also has abnormal permeability to cations, particularly sodium and potassium, likely due to the disruption of the cytoskeleton by the mutated protein.

Review Questions

1. b; 2. a; 3. a; 4. b; 5. a; 6. d; 7. a; 8. b; 9. b; 10. a; 11. d

CHAPTER 25

Case Study

- Many malarial ring forms, with multiple ring forms in individual red blood cells (RBCs), are present in the thin peripheral blood film. Many ring forms and a crescent-shaped gametocyte are also present in the thick peripheral blood film.
- The high parasitemia, the presence of multiple ring forms in individual RBCs, the crescent-shaped gametocyte on the thick film, and the absence of other parasite stages in the thin and thick peripheral blood films suggest a diagnosis of malaria due to *Plasmodium falciparum*.
- The patient had typical symptoms of malaria after a recent 3-week trip to Ghana in West Africa. Malaria is endemic in Ghana, and according to the Centers for Disease Control and Prevention,⁵² most of the malaria cases in Ghana are due to *P. falciparum*.
- The only forms of *P. falciparum* that are seen on a peripheral blood film are ring forms and gametocytes, and the latter are characteristically crescent-shaped.
- Anemia in malaria is due to direct lysis of infected RBCs during schizogony; immune destruction of infected and noninfected RBCs by macrophages in the spleen; and inhibition of erythropoiesis and ineffective erythropoiesis.

Review Questions

1. c; 2. a; 3. b; 4. b; 5. c; 6. b; 7. c; 8. c; 9. c; 10. d; 11. c

CHAPTER 26

Case Study

- The WBC can be elevated due to an underlying infection or the autoimmune response itself (inflammation). The MCV is elevated due to the reticulocytosis; the RDW is slightly elevated due to the anisocytosis and occasional schistocytes. The reticulocyte count is increased due to a surge in RBC production in the bone marrow in response to the anemia.

- In this immune process, spherocytes develop from IgG-sensitized RBCs that have had the immune complex (and a part of the cell membrane) removed by macrophages. The membranes seal and the cells become spherocytic. The red pulp of the spleen eventually entraps the spherocytes, which are less deformable, and macrophages engulf and digest them, thus shortening their life span.
- The direct antiglobulin test detected an IgG autoantibody which attached to the patient's RBCs in vivo, which is a hallmark of WAIHA. The IgG autoantibody was also detected in the serum with the antibody screen using the indirect antiglobulin test. The patient's RBCs, sensitized with IgG autoantibody, were prematurely ingested and destroyed by macrophages (extravascular hemolysis); within the macrophages hemoglobin is degraded to polypeptide chains, iron, and the protoporphyrin ring. The protoporphyrin is converted to unconjugated bilirubin and is transported to the liver where it is conjugated with glucuronic acid to form conjugated bilirubin. When there is excessive hemolysis, the liver cannot process all the excess unconjugated bilirubin that is being formed, so it accumulates in the serum. The excess conjugated bilirubin formed in the liver is excreted through the bile duct to the intestines where it is converted to urobilinogen. Because of the increased urobilinogen produced in the intestines, an increased amount is reabsorbed into the blood, and an increased amount is excreted in the urine. There is also an increase in intravascular hemolysis which liberates lactate dehydrogenase and elevates the level in serum. Free hemoglobin is also liberated and is bound by haptoglobin. The hemoglobin-haptoglobin complex is taken up and degraded by macrophages, resulting in a decrease in serum haptoglobin. When the serum haptoglobin is depleted, the excess hemoglobin accumulates in the plasma. Some is salvaged by hemopexin, but the excess is filtered by the kidney. Some hemoglobin is absorbed by the proximal tubular cells; the iron is removed and converted to hemosiderin. When the tubular cells slough off into the urine, the hemosiderin can be detected. The excess hemoglobin that is not absorbed by the tubular cells flows into the urine resulting in hemoglobinuria.
- Prednisone is a glucocorticosteroid with immunosuppressive properties, such as reducing WBC response to inflammation and production of inflammatory cytokines. When a patient with an autoimmune disorder is given prednisone, most of these inflammatory mechanisms are switched off or slowed down, which in turn reduces the body's autoimmune response. The patient probably had an acute form of WAIHA because the symptoms and severe anemia developed suddenly and there was no evidence of an underlying condition.
- With parents of the genotypes SC and AS, 25% of the offspring would have each of the following genotypes: AS, SS, AC, and SC.

Review Questions

- d; 2. b; 3. c; 4. b; 5. d; 6. b; 7. a; 8. b; 9. c; 10. a; 11. b; 12. d; 13. d; 14. b; 15. c; 16. d

CHAPTER 28

Case Study

- The family history revealed a Mediterranean ethnic background; both α - and β -thalassemia are common in the Mediterranean population. The student's mother had always been anemic, and her gallbladder "attacks" were probably caused by pigmented gallstones (calcium bilirubinate), which resulted from the mild hemolytic anemia of heterozygous β -thalassemia. A cousin on the mother's side had children with thalassemia major. Because of the family history, it is quite likely that the student has β -thalassemia minor. Note that his mother was periodically given iron therapy. It is a common mistake to treat a thalassemic individual for iron deficiency anemia, especially in areas in which thalassemia is not common in the general population, because both iron deficiency anemia and thalassemia are microcytic, hypochromic anemias.
- The student had a mild hypochromic (decreased mean cell hemoglobin concentration) and microcytic (decreased mean cell volume) anemia with target cells and basophilic stippling on his peripheral blood film. He had an elevated level of hemoglobin A₂, which is a marker for β -thalassemia minor. His serum ferritin level was within the reference interval, which ruled out a diagnosis of iron deficiency anemia.
- A microcytic, hypochromic anemia could be due to α - or β -thalassemia, Hb E disease or trait, iron deficiency anemia, or, more rarely, sideroblastic anemia (including lead poisoning) or anemia of chronic inflammation (see Figure 19-2). Iron deficiency anemia is the most common of these. Iron studies can differentiate these conditions. An incorrect presumption that a patient has iron deficiency may lead to inappropriate iron therapy or to unnecessary diagnostic procedures.
- The potential mother should be screened for β -thalassemia trait, and if she is heterozygous for a β -thalassemia gene mutation, the couple should be advised that there is a 25% chance of having a baby with β -thalassemia major (homozygous or compound heterozygous for a β -thalassemia mutation). In addition, there is a 25% chance of having a baby who is homozygous for normal β -globin genes, and a 50% chance of having a baby heterozygous for a β -thalassemia mutation (β -thalassemia trait). Molecular genetic testing of the *HBB* gene is performed for carrier detection in couples seeking preconception counseling.

Review Questions

- b; 2. a; 3. a; 4. d; 5. d; 6. d; 7. a; 8. c; 9. c; 10. c; 11. c

CHAPTER 27

Case Study

- The confirmatory test that should be performed is citrate agar electrophoresis at a pH between 6.0 and 6.2. In the citrate agar test, Hb C is separated from Hb A₂, Hb O, and Hb E, and Hb S is separated from Hb D and Hb G (see Figure 27-7).
- The characteristic morphologic feature on the peripheral blood film is a Hb SC crystal. They appear as fingerlike or quartzlike crystals of dense hemoglobin protruding from the RBC membrane.
- On the basis of the electrophoretic pattern and RBC morphology, Hb SC disease is likely.

Review Questions

- b; 2. c; 3. a; 4. d; 5. a; 6. c; 7. a; 8. c; 9. d; 10. a; 11. d; 12. b; 13. c; 14. d; 15. c

CHAPTER 29

Case Studies

Case 1

- Chronic granulomatous disease.
- Patient neutrophils are unable to form reactive oxygen species such as hydrogen peroxide.

- Aggressive treatment of infections and use of antifungal agents have greatly increased survival rates so that the majority of patients survive into adulthood.
- The majority of cases are X-linked.

Case 2

- Because of the reactive monocytosis, the blood film should be examined for possible circulating macrophages.
- On the edges of the blood film, because macrophages are very large cells.
- Circulating macrophages indicate sepsis.
- A buffy coat preparation, which concentrates nucleated cells.

Review Questions

- a; 2. d; 3. c; 4. b; 5. d; 6. c; 7. d; 8. c; 9. b; 10. c

CHAPTER 30

Case Study

- G banding utilizes Giemsa staining to differentiate chromosomes into bands for identification of specific chromosomes. The chromosomes must be pretreated with the proteolytic enzyme trypsin.
- The mutation is an example of a structural rearrangement between chromosomes 9 and 22, called the *Philadelphia chromosome*. The Philadelphia chromosome represents a balanced translocation between the long arms of chromosomes 9 and 22. At the molecular level, the gene for *ABL1*, an oncogene, joins a gene on chromosome 22 named *BCR*. The result of the fusion of these two genes is a new fusion protein.
- Fluorescence in situ hybridization (FISH) is a molecular technique that uses DNA or RNA probes labeled directly with a fluorescent nucleotide or with a hapten (e.g., dinitrophenyl, digoxigenin, or biotin). Both the probe and either metaphase or interphase cells are made single-stranded (denatured) and then hybridized together. Cells hybridized with a direct-label probe are viewed with a fluorescence microscope. If the probe was labeled with a hapten, antibodies to the hapten, carrying a fluorescent tag, are applied to the cells. Once the antibodies bind to the RNA or DNA probe, the cells can be viewed using a fluorescence microscope. FISH complements standard chromosome analysis by confirming the G-band analysis and by improving resolution, which allows for analysis at the molecular level.

Review Questions

- c; 2. d; 3. a; 4. d; 5. a; 6. c; 7. d; 8. c; 9. c; 10. b

CHAPTER 31

Case Study

- DNA isolation for the detection of inherited mutations requires whole blood collected in a lavender stopper tube containing EDTA to preserve white blood cells.
- The correct controls are present and include a positive control (Lane B), a negative control (Lane D), and a "no-DNA" control (Lane E). The no-DNA control is essential when any polymerase chain reaction (PCR) test is performed in the clinical laboratory. This control will demonstrate whether cross-contamination occurred during the setup of the PCR procedure. The no-DNA control region of the gel should lack a banding pattern, as seen in Figure 31-1. If a banding pattern is present in the no-DNA control region or this control is missing, the test must be repeated before reporting patient results.

- Bands in the patient's sample (Lane C) appear at 141, 104, and 82 bp.
- The following band sizes are expected in factor V Leiden DNA analysis:
 - Normal: 104 and 82 bp (37 bp is sometimes barely visible, as well)
 - Heterozygous: 141, 104, and 82 bp (37 bp is sometimes barely visible, as well)
 - Homozygous: 141 and 82 bp
- The three bands in the patient sample indicate that this patient is heterozygous for the factor V Leiden mutation.

Review Questions

- d; 2. b; 3. d; 4. b; 5. b; 6. c; 7. a; 8. a; 9. b; 10. b

CHAPTER 32

Case Studies

Case 1

- The lymphoid population is the most prominent. Forward scatter demonstrates small to medium-sized cells. These cells are characterized by low side scatter indicative of sparse agranular cytoplasm.
- The majority of cells express CD19, CD10, and κ light chain. There is also a small population of T cells positive for CD5 and negative for CD19 antigen.
- Prominent κ light chain expression indicates a monoclonal B-cell population that is characteristic of lymphoma.

Case 2

- The low density of CD45 antigen coupled with relatively low side scatter is characteristic of a blast population. Such a prominent blast population can only be seen in acute leukemias.
- The expression of immature markers (CD34 and HLA-DR) coupled with positivity for myeloid and megakaryoblastic antigens (CD33, CD41, and CD61) is seen in acute megakaryoblastic leukemias.

Review Questions

- c; 2. b; 3. a; 4. d; 5. b; 6. a; 7. a; 8. a; 9. c; 10. a; 11. b

CHAPTER 33

Case Study

- An elevated white blood cell (WBC) count with a left shift suggests a myeloproliferative neoplasm or a leukemoid reaction (reactive neutrophilia). However, in this patient the WBC count was extremely elevated, the left shift was rather deep (presence of promyelocytes and blasts), and basophilia was present, which suggests that a myeloproliferative neoplasm is likely present. Chronic myelogenous leukemia (CML) is the most likely cause of these laboratory findings.
- The leukocyte alkaline phosphatase (LAP) score is low in CML due to inappropriate LAP synthesis in the secondary granules, whereas LAP is elevated in bacterial infections due to activation of enzyme synthesis.
- The *BCR/ABL1* fusion gene must be identified to confirm the diagnosis of CML. *BCR/ABL1* can be demonstrated from a karyotype analysis showing the t(9;22) reciprocal translocation known as the *Philadelphia chromosome* (Chapter 30), by demonstration of the *BCR/ABL1* fusion gene using fluorescence in situ hybridization (Chapter 30), or by demonstration of the *BCR/ABL1* fusion mRNA

by qualitative reverse transcriptase polymerase chain reaction (Chapter 31). Patients who have complete blood count and differential results that resemble those in CML but test negative for *BCR/ABL1* are considered to have atypical CML, and the disorder is classified as myelodysplastic syndrome/myeloproliferative neoplasm (Chapter 34).

- Cytogenetic studies are likely to show the t(9;22) mutation.
- The t(9;22) translocation produces the *BCR/ABL1* chimeric gene, which is observed in four primary molecular forms that produce three versions of the *BCR/ABL* chimeric protein: p190, p210, and p230.
- First-line therapy for CML is the tyrosine kinase inhibitor imatinib mesylate (Gleevec). Allogeneic stem cell transplantation should be considered for all CML patients, because it is the only potentially curative treatment for CML. However, few CML patients qualify for allogeneic stem cell transplantation, because most do not meet the criteria for low risk: age younger than 40 years, disease in the chronic phase, transplantation within 1 year of diagnosis, and availability of an HLA-matched donor. For those patients who qualify for allogeneic stem cell transplantation, imatinib is used to induce remission prior to transplant, to treat minimum residual disease, and to provide rescue therapy if the transplant fails. Imatinib is continued as lifelong therapy until drug resistance is detected.
- The majority of cases of imatinib resistance result from two primary causes: acquisition of additional *BCR/ABL1* mutations and expression of point mutations in the adenosine triphosphate (ATP) binding site. Additional *BCR/ABL1* mutations can occur through the usual translocation of the remaining unaffected chromosomes 9 and 22, which converts the hematopoietic stem cell from heterozygous to homozygous for the *BCR/ABL1* mutation. A double dose of *BCR/ABL1* can also be acquired from gene duplication during mitosis and accounts for 10% of secondary mutations. An additional *BCR/ABL1* mutation will double the tyrosine kinase activity, which makes the imatinib dosage inadequate. In these cases higher dosages of imatinib will restore remission in most patients. Over 60 mutations have been identified in the ATP binding site, and these account for the remaining 50% to 90% of secondary mutations. Mutations in the ATP binding site reduce the binding affinity of imatinib, producing some level of resistance.

Review Questions

- b; 2. c; 3. d; 4. c; 5. c; 6. c; 7. b; 8. d; 9. a; 10. c

CHAPTER 34

Case Study

- The differential diagnosis of patients with pancytopenia should include megaloblastic anemia (vitamin B₁₂ or folate deficiency), aplastic anemia, liver disease, alcoholism, and myelodysplastic syndrome (MDS).
- The probable diagnosis is MDS.
- This patient's MDS should be classified as refractory anemia with ringed sideroblasts (RARS).

Review Questions

- d; 2. a; 3. b; 4. b; 5. c; 6. c; 7. a; 8. d; 9. c; 10. c

CHAPTER 35

Case Study

- Due to the presence of blasts on the peripheral blood film, the most likely diagnosis is acute leukemia. The thrombocytopenia and

anemia support that diagnosis. According to the WHO classification, $\geq 20\%$ blasts in the bone marrow is required for diagnosis of acute leukemia; an exception to this criterion are those cases that have specific genetic abnormalities (delineated in the WHO classification) that are diagnostic, regardless of blast count. Acute lymphoblastic leukemia (ALL) is more common in children. Immunophenotyping by flow cytometry determines the lineage and maturation stage of the blasts. Testing for genetic abnormalities is required for diagnosis and prognosis.

- This child has clinical and laboratory features indicative of a favorable prognosis: young age, a white blood cell count less than $20 \times 10^9/L$ (i.e., low tumor burden), and hyperdiploidy. The strongest predictor of patient outcome is the presence of certain genetic abnormalities; the immunophenotype also contributes to the prognosis.
- Hyperdiploidy carries a favorable prognosis in B-cell ALL in children.

Review Questions

- b; 2. b; 3. a; 4. d; 5. b; 6. d; 7. c; 8. c; 9. b; 10. b; 11. b; 12. b

CHAPTER 36

Case Study

- Diffuse large B-cell lymphoma (DLBCL).
- This lesion is expected to show exclusive (clonal) κ or λ light chain expression. Flow cytometry is particularly sensitive in detecting surface and cytoplasmic immunoglobulin light chains and is commonly used to confirm clonality of lymphoproliferative disorders. In addition, other pan-B-cell markers can be studied by flow cytometry (e.g., CD19, CD22, and FMC7 antigens) to demonstrate the B-cell origin of this lymphoma.
- Most often DLBCL presents as a localized disease involving a group of lymph nodes. Bone marrow involvement is rare at presentation; however, it can occur later in the course of the disease.

Review Questions

- d; 2. c; 3. d; 4. d; 5. b; 6. b; 7. b; 8. a; 9. c; 10. a

CHAPTER 37

Case Study

- Given the family history, this may be an inherited condition, although pregnancy is an independent risk factor for thrombosis.
- Thrombosis is probably caused by the deficiency of a coagulation inhibitor such as protein C, protein S, or antithrombin. It may be caused by a procoagulant gain-of-function mutation such as the factor V Leiden mutation or the prothrombin G20210A mutation.

Review Questions

- b; 2. c; 3. b; 4. d; 5. b; 6. d; 7. b; 8. c; 9. a; 10. a

CHAPTER 38

Case Study

- The combination of thrombocytopenia and prolonged prothrombin time (PT) and partial thromboplastin time (PTT) indicate probable liver disease. In the absence of a full medical history, the patient's hemarthroses and description of himself as a "bleeder" lead to the presumption of hemophilia, possibly hemophilia A. It is possible that he contracted hepatitis C from an untreated blood product. Treatment of factor concentrates for viral disease began in

1984. Prior to 1984 most hemophilia patients eventually developed hepatitis B or C from factor concentrates. Hepatitis A is also a possibility. Liver disease may be confirmed using bilirubin and liver enzyme assays.

In advanced liver disease, poor liver circulation causes pressure in the portal circulation. This enlarges the spleen (splenomegaly). The enlarged spleen sequesters and clears platelets more rapidly than normal, a condition called *hypersplenism*, which causes thrombocytopenia. In most cases, platelet function is reduced. This reduced platelet function can be demonstrated in the laboratory using platelet aggregation and is the reason for the patient's epistaxis.

Vitamin K deficiency is also a possibility. To differentiate vitamin K deficiency from liver disease, a factor V and VII activity assay is performed. In vitamin K deficiency factor VII activity is reduced but factor V activity is normal. In liver disease, both are reduced.

2. In early liver disease the vitamin K–dependent factors II (prothrombin), VII, IX, and X are produced with diminished function. This can be corrected with a trial dose of oral or intravenous vitamin K. In people with true vitamin K deficiency secondary to an altered diet, the vitamin K therapy corrects bleeding and normalizes the PT and PTT, but in liver disease vitamin K may not have a lasting effect. This is because the liver cannot process the vitamin K normally.

In addition to vitamin K therapy, thawed frozen plasma (FP) transfusion at 1 to 2 units/day is effective in supplementing the liver's production of all the coagulation factors. Cryoprecipitate may also be used to raise the fibrinogen concentration, and platelet concentrate may be used if the platelet count drops to below 50,000/ μ L and there is continued evidence of mucocutaneous bleeding.

Administration of vitamin K, FP, cryoprecipitate, and platelets does not cure liver disease; these therapies only treat the bleeding symptoms. Additional treatment may include antibiotics, antivirals, and anti-inflammatory drugs.

Review Questions

1. c; 2. d; 3. b; 4. c; 5. b; 6. b; 7. d; 8. a; 9. a; 10. c; 11. c

CHAPTER 39

Case Study

- The following tests for congenital and acquired risk factors are included in a thrombophilia profile. Results for the items with asterisks are valid only when the test is performed 10 to 14 days after termination of antithrombotic therapy or resolution of a thrombotic event.
 - Homocysteine
 - Lupus anticoagulant profile*
 - Prothrombin G20210A mutation
 - Activated protein C resistance*
 - Factor V Leiden mutation (confirmatory for activated protein C resistance)
 - Anticardiolipin antibodies by immunoassay
 - Protein C functional assay and follow-up immunoassay*
 - Protein S functional assay and follow-up immunoassay*
 - Antithrombin functional assay and follow-up immunoassay*
- The most common acquired thrombotic risk factors are antiphospholipid antibodies and lupus anticoagulant, and these are most often implicated in a thrombotic event.
- Patients with thrombotic risk factors may be instructed to avoid situations and practices that may trigger thrombosis, such as immobilization, smoking, and use of oral contraceptives or

hormone replacement therapy. They may be provided with prophylactic antithrombotic therapy at times when circumstances increasing thrombotic risk cannot be avoided, such as when undergoing orthopedic surgery.

Review Questions

1. b; 2. a; 3. d; 4. c; 5. a; 6. b; 7. a; 8. a; 9. c; 10. d; 11. d; 12. d; 13. d; 14. c

CHAPTER 40

Case Study

- Yes, the heparin is significant.
- Heparin-induced platelet aggregation assay, serotonin release assay, or enzyme-linked immunosorbent assay (ELISA) should be ordered.

An ELISA was performed to look for the presence of heparin-induced antibodies. Results gave an optical density of 0.650, with a reference interval of less than 0.400 optical density. The patient was found to have clinically significant levels of heparin-induced antibodies.

The patient underwent an above-knee amputation of her right leg. No heparin or low molecular weight heparin was used during or after the procedure. The grafting surgeries were successful, and the patient recovered.

Review Questions

1. b; 2. d; 3. b; 4. c; 5. b; 6. b; 7. b; 8. c; 9. d; 10. a

CHAPTER 41

Case Study

- Storage pool disease, aspirin-like defects, and use of antiplatelet agents such as aspirin are possibilities.
- Storage pool disease or aspirin-like defects seem most likely.
- Based on the results of the quantitative test for adenosine triphosphate release, the likely cause is dense granule storage pool disease.

These results were confirmed by the findings of electron microscopy of the patient's platelets, which revealed the absence of detectable dense granules. Because the patient's bleeding problems are due to an inherited abnormality that typically results in only mild bleeding problems, the patient was counseled to avoid antiplatelet agents, particularly aspirin, since they are known to exacerbate the bleeding problems encountered by patients with dense granule deficiency.

Review Questions

1. d; 2. a; 3. a; 4. b; 5. c; 6. c; 7. d; 8. b; 9. d; 10. a

CHAPTER 42

Case Study

- The laboratory director questioned the phlebotomist about the problem. The phlebotomist admitted that he had erroneously collected blood in a red- and gray-stoppered "tiger-top" tube and, responding to the patient's remark, had immediately poured the blood into a blue-stoppered tube for analysis. He thought the specimen would be okay because it had not clotted yet.
- The red and gray marbled stopper designates a serum separator tube. The phlebotomist poured the blood into the blue-stoppered tube before it had begun to clot; however, the activator from the

tiger-top tube shortened the clotting time on the prothrombin time (PT) test, thus causing an erroneously short PT and low international normalized ratio (INR).

- Unexpectedly short PTs during oral anticoagulant therapy are generally indicators of patient non-compliance to the drug regimen. The second most common circumstance that affects the PT is dietary changes, most often an increased intake of vitamin K-rich foods such as green leafy vegetables, liver, or avocado. In this instance the patient had been fully compliant, carefully following the prescribed dosage and timing, and her diet had not changed. These facts led the laboratory director to consider a specimen collection error.

Specimens collected in 3.2% sodium citrate may be stored for up to 24 hours at room temperature without a change in the PT. However, specimens stored at higher than 24° C deteriorate rapidly, which causes prolongation of the PT and increase in the INR. Prolonged storage at 2° to 4° C may activate factor VII, which slightly shortens the PT and slightly decreases the INR.

Many serum separator tubes contain particulate materials that hasten in vitro clotting. Core laboratory managers select these tubes to improve test result turnaround time when the required sample is serum. When blood is collected into a series of tubes that includes a blue-stoppered tube for hemostasis testing, the blue-stoppered tube should be filled first or should be filled after a tube without additives. It should not be filled immediately after filling a serum separator tube with clot activators, because the activators may carry over to the hemostasis specimen and affect test results.

In this case, an observant patient provided clues that led to identification of the pre-analytical error. The phlebotomist was carefully counseled about the effects of tube additives on hemostasis tests.

Review Questions

- c; 2. b; 3. a; 4. b; 5. a; 6. a; 7. b; 8. b; 9. b; 10. d; 11. b; 12. d; 13. d; 14. c

CHAPTER 43

Case Study

- The increase in anticoagulation could be caused by a change in diet, dietary supplements, or drugs. Any new drug that interferes with the cytochrome oxidase P-450 enzyme 2C9 pathway could reduce warfarin breakdown and excretion, and increase its effectiveness.
- Determine what has caused the change in warfarin efficacy and eliminate it if possible, adjust the warfarin dosage, or give vitamin K orally or intravenously to stop bleeding if necessary.
- The chromogenic factor X assay.

Review Questions

- c; 2. c; 3. c; 4. a; 5. d; 6. b; 7. d; 8. c; 9. b; 10. b; 11. a; 12. a; 13. a; 14. d; 15. d

CHAPTER 44

Case Study

- No. The description of the sample and the instrument flags indicating lipemia should alert the operator to a potentially invalid test result because lipemia is known to cause erroneous results on some photo-optical coagulation analyzers.
- Two options are available to negate the effect of the lipemia and obtain valid test results:
 - Remove the lipids from the plasma by high-speed centrifugation or ultracentrifugation.
 - Perform testing using an endpoint detection method that is not susceptible to lipemia in the sample, such as mechanical clot detection.
- Because the patient history includes previous surgical procedures without bleeding symptoms and there is no other indication of abnormal bleeding tendencies for this patient, it is probably safe to consider that the prolonged prothrombin time and activated partial thromboplastin time results are due to the lipemic nature of the sample. The patient would most likely not be at risk for bleeding during the surgery, and it would be anticipated that repeat testing using one of the options listed previously would yield test results within the reference interval.

Review Questions

- b; 2. d; 3. a; 4. c; 5. b; 6. c; 7. c; 8. a; 9. d; 10. a

CHAPTER 45

Case Study

- Yes, the newborn reference interval for hemoglobin is 16.5 to 21.5 g/dL and for the hematocrit is 48% to 68%.
- These values are normal for newborns. Erythrocytes of a newborn are markedly macrocytic. There may be 2 to 24 nucleated red blood cells on the first postnatal day, but they are not present by day 5. The polychromasia reflects the reticulocytosis that persists for about 4 days.
- These values are within the reference intervals for newborns. The white blood cell count of a newborn fluctuates a great deal with a reference interval of 9.0 to $37.0 \times 10^9/L$, and leukocytosis without evidence of infection is common. The differential may show an increase in neutrophils rather than the lymphocyte predominance seen after 2 weeks. In this case the neutrophils and lymphocytes were present in equal amounts, but no immature neutrophils were seen.

Review Questions

- d; 2. b; 3. a; 4. b; 5. c; 6. c; 7. d; 8. b; 9. a; 10. c

Glossary

- a- β -lipoproteinemia** Autosomal recessive disorder of lipoprotein metabolism in which lipoproteins containing apolipoprotein B (chylomicrons, very-low-density lipoproteins, and low-density lipoproteins) are not synthesized. Characterized by the presence of peripheral blood film acanthocytes and low plasma cholesterol levels.
- absolute neutrophil count (ANC)** Total of neutrophils per liter of blood. The absolute neutrophil count is calculated by multiplying the total white blood cell count by the percentage of segmented neutrophils and bands or may be counted directly using an automated hematology analyzer.
- absolute reticulocyte count (ARC)** Reticulocytes per liter. The absolute reticulocyte count is calculated by multiplying the patient's visual reticulocyte count (reticulocyte percentage, VRET%) by the red blood cell count or may be measured directly using an automated hematology analyzer.
- acanthocyte** Red blood cell with spiny projections of varying lengths distributed irregularly over its surface, associated with lipid imbalance. Contrast with the echinocyte, which has regular projections of uniform length.
- acanthocytosis** Presence of acanthocytes in the blood. Associated with abetalipoproteinemia or abnormalities of lipid metabolism, such as abnormalities occurring in liver disease.
- accuracy** Extent to which an assay result matches its true value. Accuracy is computed by comparing assay results with the results from an established reference assay or a primary standard.
- achlorhydria** Pathologic absence of free hydrochloric acid from gastric secretions following stimulation.
- acid elution slide test (Kleihauer-Betke stain)** Test for detecting fetal red blood cells (RBCs) in the maternal circulation. Blood films are immersed in an acid buffer, which causes adult hemoglobin (Hb A) to be eluted from RBCs. The film is stained, and RBCs that have fetal hemoglobin (Hb F) take up the stain.
- acquired immunodeficiency syndrome (AIDS)** Late-stage immune system suppression characterized by depletion of CD4⁺ T lymphocytes and depression of cellular immunity causing susceptibility to opportunistic infections and neoplasms. Caused by infection with human immunodeficiency virus (HIV), a retrovirus.
- acrocentric** Describes the appearance of a metaphase chromosome with the centromere near one end, which causes the q arm to be much longer than the p arm.
- acrocyanosis (Raynaud phenomenon)** Persistent symmetrical cyanosis (blotchy blue or red discoloration) in the skin of the digits, palm, wrists, and ankles, and less frequently the nose and ears, upon prolonged exposure to cold.
- activated coagulation time (ACT)** Whole-blood clotting time test often used in cardiac surgical suites. A particulate activator is added to blood, the mixture is rocked, and the interval to clotting is recorded. Employed to monitor high-dose unfractionated heparin therapy during cardiac catheterization or coronary artery bypass graft surgery.
- activated partial thromboplastin time (APTT, partial thromboplastin time, PTT)** Clot-based screening test for intrinsic coagulation that is prolonged in deficiencies of prekallikrein, high-molecular-weight kininogen, factors XII, XI, IX, VIII, X, V, and II (prothrombin), and fibrinogen. Calcium chloride, phospholipid, and negatively charged particulate activator are added to patient plasma. The interval from the addition of reagent to clot formation is recorded. The test is used to monitor unfractionated heparin therapy and to screen for intrinsic and common pathway deficiencies, specific factor inhibitors, and lupus anticoagulant.
- activated protein C (APC)** Coagulation pathway regulatory protein activated by the thrombin-thrombomodulin complex that, when bound and stabilized by protein S, hydrolyzes and inactivates factor Va and factor VIIIa.
- activated protein C resistance (APCR)** Inherited condition in which activated coagulation factor V (Va) resists activated protein C digestion, which results in an increased risk of venous thrombosis. In 90% of cases, activated protein C resistance is caused by the factor V Leiden mutation.
- activated prothrombin complex concentrate** Therapeutic plasma preparation that bypasses factor VIII activation, used to treat bleeding episodes in hemophilic patients who have developed factor VIII inhibitor. Contains activated factors II (prothrombin), VII, IX, and X. Also known as *prothrombin complex concentrate, factor eight inhibitor bypassing activity* (FEIBA; Baxter Healthcare Corporation, Deerfield, IL).
- acute** Describes diseases whose symptoms begin abruptly with marked intensity and then subside after a relatively short period.
- acute leukemia** Malignant, unregulated proliferation of hematopoietic progenitors of the myeloid or lymphoid cell lines. Characterized by abrupt onset of symptoms and, if left untreated, death within months of the time of diagnosis.
- acute myocardial infarction (AMI)** Occlusion of a coronary artery by a clot, causing ischemia and necrosis (tissue death) of surrounding heart muscle. Commonly called a *heart attack*.
- acute phase reactant** Serum protein produced by the liver whose level rises during inflammation. Examples include C-reactive protein, ferritin, and fibrinogen.
- adenopathy** Enlargement of one or more lymph nodes.
- adenosine diphosphate (ADP)** Purine nucleotide that activates platelets by binding platelet receptor P2Y₁ and P2Y₁₂. Produced by hydrolysis of adenosine triphosphate.
- adenosine triphosphate (ATP)** Purine nucleotide that stores energy in the form of high-energy phosphate bonds, releasing energy upon hydrolysis to drive metabolic reactions.
- adhesion** Property of binding or remaining in proximity; for example, attachment of platelets to surfaces such as subendothelial collagen.
- adipocyte** Fat cell; adipocytes make up adipose tissue and the yellow portion of the bone marrow.
- afibrinogenemia** Complete absence of plasma fibrinogen.
- agammaglobulinemia** Immunodeficiency characterized by an absence or extremely reduced level of plasma gamma globulin and reduced levels of immunoglobulins. Associated with increased risk of infection.
- agglutination** Cross-linking of antigen-bearing cells or particles by a specific antibody to form visible clumps.
- aggregation** Cluster or clump of similar cell types or particles; for example, attachment of platelets to other platelets, red blood cell clumping.
- agnogenic** Of idiopathic or unknown origin.
- agranulocytosis** Any condition involving decreased numbers of granulocytes (segmented neutrophils or band neutrophils).

- albinism** Hereditary condition characterized by partial or total lack of melanin pigment in the body; skin, hair, and eyes may be affected. Individuals with total albinism have pale skin that does not tan, white hair, and pink eyes. Albinism is often associated with platelet storage pool deficiency.
- Alder-Reilly anomaly** Autosomal dominant polysaccharide metabolism disorder in which white blood cells (WBCs) of the myelocytic series, and sometimes all WBCs, contain coarse azurophilic mucopolysaccharide granules.
- allele** One of two or more alternative forms of a gene that occupy corresponding loci on homologous chromosomes. Each allele encodes a certain inherited characteristic. An individual normally has two alleles for each gene, one contributed by the mother and one by the father. If both alleles are the same, the individual is homozygous, but if the alleles are different, the individual is heterozygous. In heterozygous individuals, one of the alleles may be dominant and the other recessive.
- alloantibody (isoantibody)** Antibody that is produced in response to the presence of foreign antigens; for instance, an antibody to a therapeutic coagulation factor that may render factor therapy ineffective.
- alloimmune** Producing antibodies to antigens derived from a genetically dissimilar individual of the same species.
- alloimmune hemolytic anemia** Anemia caused by antibodies stimulated by exposure to foreign red blood cell (RBC) antigens. Antibodies coat and shorten the life span of circulating RBCs. This is the basis for hemolytic disease of the newborn.
- α -granules** Platelet granules that store and release a variety of hemostasis proteins. There are 50 to 80 α -granules per platelet. In transmission electron microscopy, α -granules appear light gray, in contrast to δ -granules (dense bodies), which appear black.
- α -thalassemia** Moderate to severe inherited anemia caused by a decreased or absent production of the α -globin chains of hemoglobin.
- amyloidosis** Disease in which a waxy, starchlike glycoprotein (amyloid) accumulates in tissues and organs, impairing their function.
- analogue drugs** Drugs that are chemically similar to one another but, because of minor structural differences, may have different physiologic actions.
- anaplastic** Characterized by loss of differentiation and growth without structure or form. Anaplasia is a characteristic of cancer.
- anatomic bleeding disorder** Chronic episodic bleeding into soft tissue, joints, and body cavities. Indicates a secondary coagulopathy such as hemophilia or an acquired coagulation factor deficiency.
- anemia** Diminished delivery of oxygen to tissues, as evidenced by pallor, malaise, and dyspnea. May be caused by blood loss, decreased red blood cell (RBC) production, increased RBC destruction (shortened life span).
- aneuploid** Having a chromosome number that is not an exact multiple of the normal diploid number (46 in humans) so that there are fewer or more chromosomes than normal.
- anisocytosis** Abnormal red blood cell (RBC) morphology characterized by considerable variation in RBC volume or RBC diameter on a blood film.
- annular diaphragm** Device used in phase microscopy that, together with a phase-shifting element, produces contrast between an unstained cell and its dark background.
- anoxia** Inadequate tissue oxygenation caused by poor lung perfusion or a diminished blood supply.
- antagonist** Drug that nullifies the action of another drug or that reduces a normal cellular response. Aspirin is a platelet antagonist because it reduces platelet activation.
- antecubital fossa** Concavity opposite the elbow.
- antibody (Ab)** Specialized protein (immunoglobulin) that is produced by B lymphocytes and plasma cells when the immune system is exposed to foreign antigens from bacteria, viruses, or other biologic materials. An antibody molecule has a specific amino acid sequence in its variable region that matches it to the antigen that originally stimulated its production.
- anticardiolipin antibody (ACL, ACA)** Member of the antiphospholipid antibody family that includes anti- β_2 -glycoprotein 1 and lupus anticoagulant. An ACL antibody is an autoantibody detected in a solid-phase immunoassay system using cardiolipin as the target antigen. The chronic presence of ACL antibodies is associated with venous and arterial thrombotic disease.
- anticoagulant** Therapeutic agent that delays blood coagulation, such as heparin or warfarin, used to prevent thrombotic events in patients who are at risk. Additive to blood specimen collection tubes that prevents in vitro blood clotting such as sodium citrate.
- antigen** Molecule that the immune system recognizes as foreign and that subsequently evokes an immune response.
- antihemophilic factor (AHF)** Therapeutic concentration of coagulation factor VIII produced through chemical fractionation, immunoaffinity column, or recombinant synthesis. Antihemophilic factor is prescribed in the treatment of hemophilia A, a hereditary deficiency of factor VIII.
- antineoplastic** Chemotherapeutic agent that controls or kills cancer cells.
- antiphospholipid antibody (APL, APA)** Member of the antibody family that includes anticardiolipin, anti- β_2 -glycoprotein I, and lupus anticoagulant. APL antibodies bind phospholipid-binding proteins, such as β_2 -glycoprotein. The presence of an APL antibody is associated with venous and arterial thrombotic disease.
- antiphospholipid syndrome (APS)** Group of thrombotic disorders related to the chronic presence of an antiphospholipid antibody, such as anticardiolipin, anti- β_2 -glycoprotein I, or lupus anticoagulant. Manifestations include migraine, transient ischemic attacks, strokes, acute myocardial infarction, peripheral artery disease, venous thromboembolic disease, and spontaneous abortion.
- antithrombin (AT, antithrombin III, AT III)** Plasma serine protease inhibitor produced in the liver and activated by therapeutic heparin or vascular heparan sulfate. When activated, antithrombin controls the coagulation pathway, because it neutralizes all the serine proteases except factor VIIa, most importantly factors IIa (thrombin) and Xa.
- aperture** Optical device in a microscope substage light path that controls the diameter of the light column that reaches the specimen. The aperture may be adjusted to improve specimen clarity.
- aplasia** Failure of the normal process of cell generation and development. Bone marrow aplasia is the loss of all bone marrow cellular elements.
- aplastic anemia** Deficiency of all of the formed elements of blood, representing a failure of the blood cell-generating capacity of bone marrow.
- apoferritin** Protein component of the ferritin molecule consisting of 24 identical spherical subunits that surround iron in the ferric valence form.
- apoptosis** Natural cell death characterized by nuclear condensation and loss of cytoplasmic integrity. Apoptosis is a mechanism that prevents proliferation of dysplastic or mutated cells.
- aspirin (acetylsalicylic acid, ASA)** Acetylsalicylic acid in tablet form; irreversibly acetylates platelet cyclooxygenase and reduces platelet activation. Aspirin is used for its antithrombotic properties.
- asynchrony** Disturbance of coordination that causes processes to occur at abnormal times. In hematopoietic cell development, a difference in rate between cytoplasmic and nuclear maturation.
- atrial fibrillation (AFIB)** Uncontrolled and ineffective atrial heartbeat that affects at least 2 million U.S. citizens. Atrial fibrillation raises the risk for stroke. The risk is controlled using long-term anticoagulation therapy, such as warfarin.
- atypical lymphocytes (transformed, reactive, or variant lymphocytes)** Lymphocytes whose altered morphology includes stormy blue cytoplasm and lobular or irregular nuclei. Variant lymphocytes indicate stimulation by a virus, particularly Epstein-Barr virus, which causes infectious mononucleosis.
- Auer rod** Abnormal needle-shaped or round pink to purple inclusion in the cytoplasm of myeloblasts and promyelocytes; composed of condensed primary granules. Indicates acute myeloid leukemia.

- autoantibody** Antibody produced by an individual that recognizes and binds an antigen on the individual's own tissues.
- autoimmune** Describes an immune response in which an antibody forms to one's own tissues; for instance, antinuclear antibody in lupus erythematosus.
- autoimmune hemolytic anemia** Anemia characterized by premature red blood cell (RBC) destruction. Autoantibodies to RBC surface antigens bind the membrane, causing rapid splenic clearance and hemolysis.
- autologous** Related to self or belonging to the same organism; for example, used to describe blood that is donated by patients before surgery for the purpose of transfusion to themselves during or after surgery.
- autosomal dominant inheritance** Pattern of inheritance in which the transmission of a dominant allele on an autosome causes a trait to be expressed in heterozygotes.
- autosomal inheritance** Inheritance of traits located on non-sex-related chromosomes.
- autosomal recessive inheritance** Pattern of inheritance resulting from the transmission of a recessive allele that is not expressed in heterozygotes.
- autosome** Any of the 22 pairs of chromosomes in humans other than the sex chromosomes X and Y.
- autosplenectomy** Disappearance of the spleen through progressive fibrosis and shrinkage secondary to a hemolytic anemia such as sickle cell anemia.
- azurophilic** Having cellular structures that stain blue with Giemsa stain and red-purple with Wright stain.
- azurophilic granules** Primary cytoplasmic granules in myelocytic cells that, when stained with Wright stain, appear reddish purple. Azurophilic granules of different composition may also appear in a minority of lymphocytes.
- B cell** Any of a family of lymphocytes that produce antibodies. The end product of B-cell maturation is the plasma cell.
- Babesia** Protozoal parasite transmitted by ticks that infects human red blood cells and causes babesiosis, a malaria-like illness. The parasite is an intracellular ringlike structure 2 to 3 μm in diameter.
- band neutrophil (band)** Immediate precursor of the mature segmented neutrophil. Band neutrophils have a nonsegmented, usually curved, nucleus and are present in the bone marrow and peripheral blood.
- bartonellosis** Acute febrile infection caused by the bacterium *Bartonella bacilliformis*, which is transmitted by the bite of a sandfly. The first stage of the disease is associated with severe hemolytic anemia.
- base pair** Pair of nucleotides in the complementary strands of a DNA molecule that interact through hydrogen bonding across the axis of the DNA helix. One of the nucleotides in each pair is a purine (either adenine or guanine), and the other is a pyrimidine (either thymine or cytosine). Adenine always pairs with thymine, and guanine always pairs with cytosine.
- basophil (baso)** Granulocytic white blood cell characterized by cytoplasmic granules that stain bluish black when exposed to a basic dye. Cytoplasmic granules of basophils are of variable size and may obscure the nucleus.
- basophilia** Abnormal increase of basophils in the blood.
- basophilic normoblast (prorubricyte)** Second identifiable stage in bone marrow erythrocytic maturation; it is derived from the pronormoblast (rubriblast). Typically 10 to 15 μm in diameter, the basophilic normoblast (prorubricyte) has cytoplasm that stains dark blue with Wright stain.
- basophilic stippling** Barely visible dark blue to purple granules evenly distributed within a red blood cell stained with Wright stain. Composed of precipitated ribosomal protein and RNA.
- Bence Jones protein** Protein found almost exclusively in the urine of patients with multiple myeloma, consisting of the light chain of the abnormal immunoglobulins produced.
- benign** Noncancerous or nonmalignant.
- Bernard-Soulier syndrome (BSS)** Mild to moderate mucocutaneous bleeding disorder caused by one of a series of mutations to platelet glycoprotein Ib (GP Ib) or GP IX, part of the GP Ib/IX/V von Willebrand factor receptor complex. The disorder is a defect of platelet adhesion.
- β_2 -glycoprotein I (β_2 -GPI)** Plasma globulin that is a target of the antiphospholipid antibody anti- β_2 -glycoprotein I.
- β -thalassemia** Inherited anemia caused by diminished synthesis of the β -globin chains of hemoglobin.
- β -thromboglobulin (β -TG)** Heparin-neutralizing protein that is stored and secreted by platelet α -granules.
- bilirubin** Gold-red-brown pigment, the main component of bile and a major metabolic product of the heme portion of hemoglobin, released from senescent red blood cells. Elevated bilirubin imparts a gold color to plasma and urine and may indicate hemolytic anemia, liver disease, or bile duct occlusion.
- bilirubinemia (icterus, hyperbilirubinemia)** Excess bilirubin in plasma. It imparts a gold color to plasma and may indicate hemolytic anemia, liver disease, or bile duct occlusion.
- 2,3-bisphosphoglycerate (2,3-BPG, 2,3-diphosphoglycerate, 2,3-DPG)** Product of red blood cell glycolysis that is generated in the Rapoport-Luebering shunt. 2,3-BPG is one of the main regulators of oxygen uptake and delivery by hemoglobin. 2,3-BPG decreases hemoglobin's affinity for oxygen, which enables it to more readily release oxygen to the target tissues.
- blast** Earliest, least differentiated stage of hematopoietic maturation that can be identified by its morphology in a Wright-stained bone marrow smear; for example, myeloblast, pronormoblast (rubriblast), lymphoblast.
- bleeding time (BT)** Time interval required for blood to stop flowing from a puncture wound 2 mm long and 1 mm deep on the volar surface of the forearm. Largely of historical interest, the bleeding time test was performed to evaluate vascular and platelet function.
- Bohr effect** Effect of carbon dioxide and hydrogen ions on the affinity of hemoglobin for oxygen. Increasing carbon dioxide and hydrogen ion concentrations (lower pH) decrease oxygen saturation; decreasing concentrations increase oxygen saturation.
- bone marrow** Gelatinous red and yellow tissue filling the medullary cavities of bones. Red marrow is found in most bones of infants and children and in the ends of long bones and the cavities of flat bones in adults. Diagnostic marrow specimens are collected from the anterior or posterior iliac crest or sternum in adults. Fatty yellow marrow is found in the medullary cavity of most adult long bones.
- bone marrow aspirate specimen** A 1- to 1.5-mL aliquot of gelatinous red marrow obtained by passing a needle into the marrow cavity and applying negative pressure. The aspirate specimen is spread as a smear on a microscope slide, stained, and examined for hematologic or systemic disease. The aspirate specimen provides for analysis of individual cell morphology.
- bone marrow biopsy specimen** A 1- to 2-cm cylinder of gelatinous red marrow obtained by passing a biopsy cannula into the marrow cavity, rotating, and withdrawing. The cylinder is fixed in formalin, sectioned, stained, and examined for hematologic or systemic disease. The biopsy specimen provides for analysis of bone marrow architecture.
- buffy coat** Gray-white layer of white blood cells (WBCs) and platelets that accumulates at the red blood cell-plasma interface when a tube of anticoagulated blood is allowed to stand or is centrifuged. The buffy coat may be used to harvest WBCs for microscopic analysis when the WBC count is low, and an enlarged buffy coat may indicate leukemia.
- Burkitt lymphoma** Lymphatic solid tissue tumor composed of mature B lymphocytes with a characteristic morphology, called *Burkitt cells*. Burkitt cells appear in lymph node biopsies, bone marrow, and occasionally in peripheral blood and have dark blue cytoplasm with multiple vacuoles creating a "starry sky" pattern.
- burst-forming unit (BFU)** Early hematopoietic progenitor cell stages of the erythroid and megakaryocytic cell lines characterized by their

- tissue culture growth pattern in which large colonies are produced. Contrast with the more differentiated colony-forming units, which produce smaller colonies.
- C banding** In cytogenetic analysis, a specialized Giemsa stain technique employing first an acid and then a basic buffer, which highlights the centromeres of chromosomes. The stained centromere is the C band, which helps to identify the chromosome.
- Cabot rings** Threadlike structures that appear as purple-blue loops or rings in Wright-stained red blood cell cytoplasm. They are remnants of mitotic spindle fibers that indicate hematologic disease such as megaloblastic or refractory anemia.
- calibrator (secondary standard)** Preserved material in which the analyte concentration has been assigned by reference to a primary standard or by controlled reference assays in expert laboratories. Calibrators are used for assays in which there are no primary standards, such as blood cell counts or coagulation assays.
- carboxyhemoglobin** Hemoglobin that has bound carbon monoxide, which prevents normal oxygen exchange. Carboxyhemoglobin imparts a cherry-red color to venous blood, and its reduced oxygen capacity is the basis for carbon monoxide poisoning.
- carcinoma** Malignant neoplasm of epithelial cell origin that invades surrounding tissue and may metastasize to distant regions of the body.
- cell membrane** Cell surface composed of two layers of phospholipids intermixed with cholesterol and a variety of specialized glycoproteins that support cell structure, signaling, and ion transport.
- cellular immunity (cell-mediated immunity, CMI)** Immune response initiated and mediated by T-lymphocyte secretions called *cytokines*, natural killer cells, and macrophages; the mechanism of acquired immunity characterized by the dominant role of the T lymphocytes. Cellular immunity is the basis for graft rejection, delayed hypersensitivity, and responses to viral infections and tumors.
- centriole** Cylindrical organelle composed of microtubules. Two centrioles typically orient perpendicular to each other forming the centrosome, located near the nucleus. During mitosis they replicate and move to opposite ends of the cell where they bind to spindle fibers that attach to the centromeres of chromosomes and effect their movement during metaphase.
- centromere** Constricted portion of a chromosome that attaches to a spindle fiber to effect movement during metaphase. Centromeres are categorized by their location as acrocentric (near one end), metacentric (near the center), or submetacentric (off center).
- cerebrospinal fluid (CSF)** Fluid that flows through and protects the four ventricles of the brain, the subarachnoid spaces, and the spinal canal. CSF is derived from plasma and is the site of bacterial and viral infections called *meningitis* or *encephalitis*. CSF is sampled by lumbar puncture.
- cerebrovascular accident (CVA)** Stroke; occlusion of an artery of the brain or brain hemorrhage resulting in necrosis of brain tissue and loss of function.
- Charcot-Leyden crystals** Crystalline structures that are shaped like narrow double pyramids and are found in the sputum of asthma patients and the feces of dysentery patients. Formed from the granules of disintegrating eosinophils.
- Chédiak-Higashi anomaly** Autosomal recessive disorder characterized by partial albinism, photophobia, susceptibility to infection, and the presence of giant blue granules in the cytoplasm of Wright-stained white blood cells and platelets.
- chelation** Chemical formation of a ring-shaped molecular complex in which a metal ion is covalently bound. Chelating agents such as ethylenediaminetetraacetic acid (EDTA) or sodium citrate trap calcium ions and are used as blood specimen anticoagulants. Chelators are also used to treat lead poisoning or iron overload.
- chemotaxis** Cellular movement toward or away from a chemical stimulus. Characteristic of neutrophils and monocytes, whose phagocytic activity is influenced by chemical factors released by invading microorganisms.
- chemotherapy** Treatment of neoplastic disease (cancer) by chemical agents.
- chromogen** Chemical that absorbs light and produces color. Chromogens are used in laboratory analysis by spectrophotometry.
- chromophore** The portion of a molecule that absorbs incident light and emits colored light, usually as a result of the presence of 10 or more double bonds or aromatic rings. Chromophores are the colored portions of chromogens and are synthesized in molecules to provide measurable color in laboratory assays.
- chromosome** Threadlike nuclear structure composed of condensed DNA that transmits genetic information. In humans there are 46 chromosomes, including 22 homologous pairs of autosomes and 1 pair of sex chromosomes, X and Y.
- chronic** Persisting over a long period of time, often for the remainder of a person's life.
- chronic leukemia** Malignant, unregulated proliferation of myelocytic or lymphocytic cells that appear at several stages of differentiation in peripheral blood. Characterized by slow onset and progression of symptoms.
- Clinical and Laboratory Standards Institute (CLSI)** Global nonprofit agency that uses consensus to develop and publish healthcare guidelines and standards.
- Clinical Laboratory Improvement Amendments (CLIA) of 1988** Law establishing the Clinical Laboratory Improvement Amendments Committee (CLIA), which sets and enforces standards for quality testing in the clinical laboratory.
- clone** Group of genetically identical cells derived from a single common cell through mitosis.
- Clostridium perfringens*** Anaerobic gram-positive bacteria that cause gangrene in humans. Symptoms of gangrene include intravascular hemolysis and thrombosis.
- cluster of differentiation (CD)** Cell surface membrane receptors or markers used to characterize cells by their functions. CD profiles are used in flow cytometry to identify cell types. CDs are used in hematology to identify cell clones associated with lymphoid and myelogenous leukemias and lymphomas.
- coagulation** Series of enzymatic reactions beginning with activation of factor VII by tissue factor (extrinsic/in vivo) or factor XII by a negatively charged surface (intrinsic/in vitro) and proceeding through the common pathway to the formation of an insoluble fibrin clot.
- coagulation cascade** Series of enzymatic reactions beginning with activation of factor VII by tissue factor (extrinsic/in vivo) or factor XII by a negatively charged surface (intrinsic/in vitro) and proceeding through the common pathway to the formation of an insoluble fibrin clot.
- coagulation factors** Plasma proteins, also called *procoagulants*, that circulate as inactive forms. When activated in the process of coagulation, procoagulants participate in the coagulation cascade to form a fibrin clot.
- codocyte (target cell)** Poorly hemoglobinized red blood cell (RBC) that appears in hemoglobinopathies, thalassemia, and liver disease. In a Wright-stained peripheral blood film, hemoglobin concentrates in the center of the RBC and around the periphery, causing the cell to resemble a "bull's-eye."
- coefficient of variation (CV, percent CV, CV%)** Statistical measure of the deviation of a variable from its mean divided by the mean, usually expressed as a percentage.
- colchicine** Alkaloid that blocks microtubule formation and prevents cell division. Used in cytogenetic studies to arrest mitosis in metaphase so that chromosomes may be karyotyped. Colchicine is also a component of a drug used to treat gout. Colcemid is the trademark for a colchicine derivative used in preparing chromosomes for karyotyping.
- cold agglutinin** IgM autoantibody specific for red blood cell surface membrane antigens usually of the Ii system that typically reacts at temperatures below 30° C.
- cold agglutinin disease** Acquired autoimmune hemolytic anemia resulting from red blood cell (RBC) agglutination by IgM autoantibodies that react with RBCs at temperatures above 30° C.

- Patients with cold agglutinin disease experience *Raynaud phenomenon*, characterized by pallor, cyanosis, and pain in the fingers, palms, wrists, and ankles after exposure to cold.
- colony-forming unit (CFU)** Hematopoietic progenitor cells that are derived from the pluripotential hematopoietic stem cell and give rise to the different cell lineages of the bone marrow. Named because of their ability to form colonies in tissue culture.
- colony-forming unit–granulocyte, erythrocyte, monocyte, and megakaryocyte (CFU-GEMM)** Hematopoietic progenitor cell capable of differentiating into the granulocytic (myelocytic), erythrocytic (normoblastic), monocytic, or megakaryocytic cell lines.
- colony-stimulating factor (CSF)** Cytokine that promotes the division and differentiation of hematopoietic cells.
- common coagulation pathway** The steps in the coagulation cascade from the activation of factor X through the conversion of fibrinogen to fibrin. The common pathway begins at the junction of the intrinsic and extrinsic pathways and involves factors X, V, and II (prothrombin) and fibrinogen, in order of reaction.
- complement (C)** System of at least 20 serum enzymes. The complement system responds to antigen-antibody reaction to stimulate white blood cell chemotaxis, generate inflammation, and cause red blood cell lysis.
- condenser** Substage microscope device that focuses light on the slide-mounted specimen to promote visual clarity.
- confidence interval (CI)** Range of values expected to contain the measured value (parameter) with a predetermined degree of statistical confidence. For instance, the 95% confidence interval is expected to include 95% of all values of a parameter measured in a normal population, which corresponds closely to ± 2 standard deviations.
- congenital** Describes a condition that exists at, and presumably before, birth. Often refers to a hereditary condition.
- Coombs test (direct antiglobulin test, DAT)** Screening procedure in which antihuman globulin is used to detect antibodies and complement bound to red blood cells in vivo.
- coronary artery bypass grafting (CABG)** Cardiac surgery usually requiring cardiopulmonary bypass and extracorporeal circulation in which occluded sections of coronary arteries are replaced with grafts taken from nearby arteries, for example, the internal mammary artery.
- corrected reticulocyte count** Calculation performed to correct the visual reticulocyte count for specimens with a hematocrit below 45% to the equivalent reticulocyte count at a hematocrit of 45%. In anemia, the visual reticulocyte percentage is misleadingly elevated because whole blood contains fewer red blood cells relative to reticulocytes.
- Coumadin (warfarin)** Vitamin K antagonist used as an anticoagulant to prevent thrombosis in people with atrial fibrillation, venous thromboembolism, or cardiac insufficiency. Also used prophylactically after orthopedic surgery. Warfarin suppresses vitamin K and reduces the activity of the vitamin K–dependent coagulation factors II (prothrombin), VII, IX, and X.
- cryoglobulin** Any of numerous serum globulins, typically immunoglobulins, that precipitate at around 4° C and become resuspended at 37° C.
- cryoprecipitate (CRYO)** Therapeutic agent rich in fibrinogen, factor VIII, and factor XIII, used to treat bleeding disorders in fibrinogen deficiency, factor XIII deficiency, and hemorrhagic trauma. Cryoprecipitate is collected from human plasma that has been frozen and slowly thawed.
- cyanosis** Bluish discoloration of the skin, sclera, and mucous membranes caused by poor tissue oxygenation. Usually a sign of anemia.
- cytochemical analysis** Use of specialized stains to detect cellular enzymes and other chemicals in peripheral blood films and bone marrow aspirate smears. Used to differentiate hematologic diseases, especially leukemias.
- cytogenetics** Branch of genetics devoted to the laboratory study of visible chromosome abnormalities, such as deletions, translocations, and aneuploidy.
- cytokine** Cellular product that influences the function or activity of other cells. Cytokines include colony-stimulating factors, interferons, interleukins, and lymphokines.
- cytomegalovirus (CMV)** Group of DNA viruses of the family Herpesviridae. CMV infection is asymptomatic in adults but can be transmitted to a fetus in utero to cause a potentially fatal infection or reduced intelligence. CMV can be transmitted by blood transfusions and is detected using molecular diagnostic techniques.
- cytopenia** Reduced cell count in one or more of any blood cell line—red blood cells, white blood cells, or platelets.
- cytosol** Fluid portion of the cytoplasm, less granules and organelles, as separated by ultracentrifugation.
- cytotoxic** Describes a compound or agent that destroys or damages cells.
- dacryocyte (teardrop cell)** Red blood cell with a single pointed extension, resembling a teardrop. Dacryocytes are often seen in the myeloproliferative neoplasm termed *myelofibrosis with myeloid metaplasia*.
- D-dimer (D:D, D-D)** One of the fibrin degradation products. D-dimer is composed of two fibrin D fragments covalently joined by the enzymatic action of factor XIII. The D-dimer assay is used to rule out venous thromboembolic disease and disseminated intravascular coagulation, and may be used to monitor the efficacy and length of warfarin therapy.
- deep vein thrombosis (DVT, deep venous thrombosis)** Pathologic formation of a clot in a deep leg vein such as the femoral vein. A manifestation of venous thromboembolic disease that is associated with a number of acquired or congenital thrombotic risk factors, deep vein thrombosis raises the risk of a pulmonary embolus.
- II-dehydrothromboxane B₂ (II-DHT)** Measurable urine product of the platelet cyclooxygenase (eicosanoid) activation pathway. Employed clinically to measure in vivo platelet activation and aspirin resistance.
- delayed hemolytic transfusion reaction** Hemolysis that occurs days or weeks after a blood transfusion, caused by an anamnestic response to the transfused red blood cells in a patient alloimmunized from a previous pregnancy or transfusion.
- δ check** Quality control process in which a current analyte result is compared with the result for the same analyte from the previous specimen from the same patient.
- dense granule (dense body, δ-body, δ-granule)** Platelet organelle that contains and secretes the small molecules adenosine diphosphate, adenosine triphosphate, serotonin, calcium (Ca²⁺), and magnesium (Mg²⁺). There are two to seven dense granules per platelet, and they are named by their opaque appearance in transmission electron microscopy.
- deoxyhemoglobin** Hemoglobin that is not combined with oxygen, formed when oxyhemoglobin releases its oxygen to the tissues.
- deoxyribonucleic acid (DNA)** Double-stranded, helical nucleic acid that carries genetic information. DNA is composed of nucleotide sequences with four repeating bases: adenine, cytosine, guanine, and thymine. During mitosis, DNA condenses to form chromosomes.
- des-γ-carboxy coagulation factors and coagulation control proteins** Coagulation cascade procoagulants II, VII, IX, and X; and control proteins C, S, and Z that require vitamin K–catalyzed γ-carboxylation of glutamic acid. During anticoagulant therapy with warfarin (Coumadin), which suppresses vitamin K, these des-γ-carboxylated proteins cannot participate in coagulation.
- desquamation** Shedding of epithelial elements, chiefly of the skin, in scales or sheets.
- Diamond-Blackfan anemia (DBA, congenital pure red cell aplasia)** Rare congenital anemia due to mutations in DNA repair genes, and evident in the first 3 months of life. Anemia is severe and erythropoietin resistant; the reticulocyte count does not rise. Platelet and white blood cell counts are normal.
- diapedesis** Outward passage of white blood cells through intact vessel walls.

- differential white blood cell count** Review and tabulation of 100 to 200 white blood cells (WBCs) in a stained blood film. The different types of WBCs are counted and reported as absolute counts or percentages of total WBCs. In automated hematology analyzers, the differential WBC count is accomplished by counting thousands of WBCs using various technologies.
- dilute Russell viper venom time (DRVVT)** Coagulation test performed like a prothrombin time assay. Russell viper venom activates the common coagulation pathway at the level of factor X. In the DRVVT assay, the reagent is diluted 1:500. DRVVT is prolonged by lupus anticoagulant, and the test is used routinely in screening for this antibody.
- diploid** Having two sets of chromosomes, as normally found in nuclei of somatic cells. In humans, the normal diploid number is 46.
- direct antiglobulin test (DAT, Coombs test)** Screening procedure in which antihuman globulin is used to detect antibodies and complement bound to red blood cells *in vivo*.
- direct thrombin inhibitors (DTIs)** Class of intravenous therapeutic anticoagulants including argatroban, dabigatran, and bivalirudin that suppress coagulation by directly neutralizing thrombin. DTIs are used in place of heparin when heparin-induced thrombocytopenia with thrombosis is suspected. DTI therapy may be monitored using the partial thromboplastin time.
- disseminated intravascular coagulation (DIC)** Uncontrolled activation of thrombin and consumption of coagulation factors, platelets, and fibrinolytic proteins secondary to many initiating events, including infection, inflammation, shock, and trauma. Most commonly evidenced by diffuse mucocutaneous bleeding.
- Döhle bodies** In Wright-stained peripheral blood films, gray to light blue round or oval inclusions composed of ribosomal RNA found singly or in multiples near the inner membrane surface of granulocyte cytoplasm.
- dominant** Denoting an inherited allele whose trait is expressed whenever the gene is present, even in heterozygotes.
- Donath-Landsteiner (D-L) autoantibody** IgG autoantibody with anti-P specificity that binds red blood cells at temperatures below 20° C and causes hemolysis at 37° C. Donath-Landsteiner antibody causes hemolysis in patients with paroxysmal cold hemoglobinuria.
- Down syndrome** Congenital group of physical, mental, and functional abnormalities including distinctive facial features, congenital heart disease, muscular hypotonia, and mental retardation, all associated with trisomy 21. Approximately 10% of infants with Down syndrome are born with or develop transient myeloproliferative disease (TMD), which resembles chronic myelogenous leukemia but resolves spontaneously. Within a few weeks of TMD resolution, the child has a 10% to 30% chance of developing acute myeloid or acute lymphoblastic leukemia.
- drepanocyte (sickle cell)** Abnormal crescent-shaped red blood cell containing hemoglobin S, characteristic of sickle cell anemia.
- drug-induced hemolytic anemia** Hemolytic anemia caused directly by a drug or secondary to an antibody-mediated response stimulated by the drug.
- dry tap** Term used when an inadequate sample of bone marrow fluid is obtained during bone marrow aspiration. Dry taps occur when the marrow is packed, as in chronic myelogenous leukemia, or when it is fibrotic, as in myelofibrosis with myeloid metaplasia.
- duodenum** Proximal portion of the small intestine adjacent to the stomach.
- dyscrasia** Disorder of a hematologic cell line or lines.
- dyserythropoiesis** Deranged erythropoiesis producing cells with abnormal morphology; usually applied to congenital dyserythropoietic anemia and myelodysplastic syndrome.
- dysfibrinogenemia** Presence in the plasma of structurally abnormal fibrinogen; often a result of liver disease, occasionally congenital.
- dysmegakaryopoiesis** Defective megakaryocytic production and maturation characterized by cells with abnormal morphology and increased or decreased megakaryocyte counts.
- dysmyelopoiesis** Defective myelocytic production and maturation characterized by cells with abnormal morphology; often applied to myelodysplastic syndromes.
- dysplasia** Abnormal growth pattern; for example, enlarged skull in chronic anemia. Abnormal cervical epithelial cell histopathologic features.
- dyspnea** Difficult or painful breathing.
- ecchymosis** Hemorrhagic spot, 1 cm or larger in diameter, in the skin or mucous membranes, typically forming an irregular blue or purplish patch. Also known as a *bruise*.
- echinocyte (burr cell, crenated red blood cell)** Red blood cell (RBC) with short, equally spaced, spiny projections. Burr cells are found in uremia and pyruvate kinase deficiency, and observed in all fields of a peripheral blood film. Crenated RBCs are formed by cellular dehydration (drying artifact), and not observed in all fields.
- eclampsia** Potentially life-threatening disorder during pregnancy characterized by hypertension, generalized edema, proteinuria, and convulsions.
- edema** Accumulation of excess serous fluid in a fluid compartment or tissue.
- effusion** Seepage and accumulation of plasma-derived fluid into a body cavity from blood vessels as a result of blood vessel damage or hydrostatic pressure.
- electronic impedance** Opposition to the flow of electrical current. The impedance principle of cell counting is based on the detection and measurement of changes in electrical resistance produced by cells as they transverse a small aperture in a conducting solution.
- electrophoresis** Separation and identification of proteins, nucleic acids, and hemoglobin types based on their relative rates of migration through agarose or polyacrylamide gel in an applied electrical field. Depending on the component, the rate of migration may be based on molecular mass and/or net charge.
- elliptocytes (ovalocytes)** Oval red blood cells seen in the peripheral blood in the membrane disorder hereditary elliptocytosis. May be found in low numbers in healthy states and in other anemias such as iron deficiency and thalassemia.
- elliptocytosis (ovalocytosis)** Hereditary hematologic disorder characterized by the presence of elliptocytes; often asymptomatic but may be associated with slight anemia.
- Embden-Meyerhof pathway (EMP, glycolysis)** A series of enzymatically catalyzed reactions by which glucose and other sugars are metabolized to yield lactic acid (anaerobic glycolysis) or pyruvic acid (aerobic glycolysis). Metabolism releases energy in the form of adenosine triphosphate.
- embolism** Pathologic event in which an embolus (foreign object) travels through the bloodstream, becomes lodged in an artery, and obstructs blood flow. The embolus is often a blood clot, but it may be a fat globule, air bubble, piece of tissue, or clump of bacteria. Emboli occlude an artery and cause tissue ischemia, necrosis, and loss of function. A pulmonary embolism is an embolus in a lung artery and is often fatal.
- endoplasmic reticulum (ER)** Extensive network of membrane-enclosed tubules in the cytoplasm of cells. Rough endoplasmic reticulum is rich in ribosomes that synthesize proteins and provides a pathway for transport of membrane-bound protein through the cytoplasm.
- endothelial cells** Cell layer that lines the inner surface of all blood vessels. Intact endothelial cells prevent thrombosis because they present a smooth, nonactivating surface and secrete antiplatelet and anticoagulant substances. Injured endothelial cells promote clotting through expression of tissue factor and secretion of coagulation-promoting factors, such as von Willebrand factor.
- enterocytes** Epithelial cells that form the inner lining of the intestine. Enterocytes absorb nutrients from the intestinal lumen and transport them to the portal circulation.
- eosinophil** Granulocyte with large uniform cytoplasmic granules that stain orange to pink with Wright stain. Granules usually do not obscure the segmented nucleus.

- eosinophilia** Increase in the blood eosinophil count that is associated with allergies, parasitic infections, or hematologic disorders.
- epiphyses** Ends of long bones that normally contain hematopoietic tissue.
- epistaxis** Hemorrhage from the nose; a nosebleed that requires intervention.
- Epstein-Barr virus (EBV)** Herpesvirus that causes infectious mononucleosis and leads to the appearance of variant lymphocytes.
- erythroblastosis fetalis (hemolytic disease of the fetus and newborn, HDFN)** Alloimmune anemia caused by maternal immunoglobulin G antibody that crosses the placenta and binds fetal red blood cell antigens inherited from the father; for instance, maternal anti-A with fetal A antigen. The disorder is characterized by hemolytic anemia, hyperbilirubinemia, and extramedullary erythropoiesis.
- erythrocyte (red blood cell, RBC)** Nonnucleated biconcave disk-shaped peripheral blood cell containing hemoglobin. Its primary function is oxygen transport and delivery to tissues.
- erythrocyte sedimentation rate (ESR)** Distance that red blood cells fall in a column of anticoagulated blood in a specified time period. Elevated sedimentation rates are not specific for any disorder but indicate the presence of inflammation.
- erythrocytosis** Increase in the red blood cell count in peripheral blood.
- erythroleukemia (Di Guglielmo disease, M6)** Acute malignancy characterized by a proliferation of erythroid and myeloid precursors in bone marrow, with erythroblasts with bizarre lobulated nuclei and abnormal myeloblasts in peripheral blood.
- erythron** Total mass of red blood cells circulating in the peripheral blood and their bone marrow precursors.
- erythropoiesis** Bone marrow process of red blood cell production.
- erythropoietin (EPO)** Glycoprotein hormone synthesized primarily in the kidneys and released into the bloodstream in response to hypoxia. The hormone acts to stimulate and regulate the bone marrow production of red blood cells.
- essential thrombocythemia** Myeloproliferative neoplasm characterized by marked thrombocytosis and dysfunctional platelets. Patients may experience bleeding or thrombosis.
- etiology** Causes or origin of a disease, for instance, genetic factors, infection, toxins, or trauma. Contrast with *pathogenesis*, which is the physiologic and biochemical mechanisms by which a disease progresses.
- euchromatin** Portion of DNA that is active in gene expression and stains lightly with Wright stain.
- euglobulin** In the euglobulin lysis assay, the fraction of treated plasma that contains fibrinogen, plasmin, plasminogen, and plasminogen activators, but lacks fibrinolytic inhibitors.
- exogenous** Originating from outside the body or produced by external causes; used, for example, to describe a disease caused by a bacterial or viral agent foreign to the body.
- exon** Portion of a DNA molecule that becomes translated to form a protein product. Exons and introns are transcribed from DNA to heteronuclear RNA, where the introns are excised to form messenger RNA.
- extramedullary hematopoiesis (myeloid metaplasia)** Production of blood cells outside the bone marrow, such as in the spleen, liver, or lymph nodes. Extramedullary hematopoiesis usually occurs in response to bone marrow fibrosis and loss of bone marrow hematopoiesis.
- extranodal** Located outside a lymph node.
- extravascular hemolysis** Destruction of red blood cells outside of a blood vessel, typically by splenic macrophage phagocytosis. Also called *macrophage-mediated* hemolysis.
- extrinsic coagulation pathway** Primary *in vivo* coagulation pathway. Exposure of tissue factor activates factor VII. Factor VIIa activates factors IX and X, which triggers the common pathway of coagulation and formation of fibrin.
- exudate** Fluid that has effused into a body cavity in association with bacterial or viral infections, malignancy, pulmonary embolism, or systemic lupus erythematosus. An exudate is typically cloudy, because it contains cells and concentrated protein.
- factor assay** Clot-based or chromogenic assay for specific coagulation factor activity.
- factor V Leiden mutation** Substitution of arginine with glutamine at position 506 in the factor V protein. The resultant factor V resists digestion by activated protein C. The factor V Leiden mutation results in increased thrombin production and is a thrombosis risk factor.
- Fanconi anemia** Autosomal recessive or X-linked aplastic anemia manifesting in childhood or early adult life. Characterized by mental retardation, absence of the thumb and radius, small stature, hypogonadism, and patchy brown discoloration of the skin.
- favism** Acute hemolytic anemia caused by ingestion of fava beans or inhalation of the pollen of the plant. Usually occurs in individuals with an inherited deficiency of glucose-6-phosphate dehydrogenase in red blood cells.
- ferritin** Iron-apoferritin complex, a major form in which iron is stored in the liver.
- ferrokinetics** Study of iron metabolism, including the movement of iron among the storage, transport, and functional iron compartments.
- fibrin** Fibrillar protein produced by the action of thrombin on fibrinogen in the clotting process. Fibrin is responsible for the semisolid character of a blood clot.
- fibrin degradation products (FDPs, fibrin split products, FSPs)** Fibrin fragments X, Y, D, and E and D-dimer produced by the action of fibrin-bound plasmin during fibrinolysis.
- fibrinogen** Plasma glycoprotein that is converted to fibrin by thrombin digestion.
- fibrinolysis** Continual process of digestion of fibrin by bound plasmin that has been activated by bound plasminogen activator. Fibrinolysis is the normal mechanism for the removal of fibrin clots. Fibrin is digested into fragments X, Y, D, and E and D-dimer.
- fibroblast** Connective tissue cell that differentiates into numerous cells, which comprise the fibrous tissue of the body, for instance, tissue in the walls of arteries.
- Fitzgerald factor (high-molecular-weight kininogen, HMWK)** Member of the kinin inflammatory system that is digested and activated by kallikrein to form bradykinin. Fitzgerald factor is one of the *in vitro* contact activators of the coagulation system, which also include prekallikrein and factor XII.
- Fletcher factor (prekallikrein, PK, pre-K)** Member of the kinin inflammatory system that forms active kallikrein upon digestion by kininogen. Fletcher factor helps to trigger *in vitro* coagulation contact activation. Fletcher factor deficiency prolongs the partial thromboplastin time but has no clinical consequence.
- flow cytometer** Instrument in which cells suspended in fluid flow one at a time through a focused beam of light. The light is scattered in patterns characteristic of the cells and their components. A sensor detecting the scattered or emitted light measures the size and molecular characteristics of individual cells.
- fluorescence in situ hybridization (FISH)** Laboratory technique in which fluorescence-labeled nucleic acid probes hybridize to selected DNA or RNA sequences in fixed tissue. FISH allows for the visual microscopic detection of specific polymorphisms or mutations such as *BCR/ABL* in cell or tissue specimens.
- fluorophore** Portion of a molecule that absorbs incident light and emits fluorescent light. Fluorophores are synthesized in molecules to provide measurable fluorescence in laboratory assays.
- free erythrocyte protoporphyrin (FEP)** Porphyrin precursor of heme that is present in low concentrations in normal red blood cells (RBCs). Elevated RBC concentrations indicate iron deficiency or impaired iron insertion.
- French-American-British (FAB) classification** International classification system for acute leukemias, myeloproliferative neoplasms, and myelodysplastic syndromes developed in the 1970s and 1980s. Still in use, although it is being displaced by the World Health Organization classification.
- fresh frozen plasma (FFP)** Plasma that is separated by centrifugation from whole-blood donations and frozen within 8 hours of collection. FFP contains all of the plasma procoagulants and

- control proteins, including the labile factors V and VIII. FFP is used for replacement therapy in acquired multiple-factor deficiencies, or in single-factor deficiencies when factor concentrates are not available.
- G banding (GTG banding)** In cytogenetic analysis, a procedure in which metaphase chromosomes are treated with trypsin and then stained with Giemsa dye. The areas rich in adenine-thymine, called G+, stain intensely, whereas the areas rich in guanine-cytosine (G-) stain more lightly. The G+ bands correspond with Q bands in Q banding. Banding patterns are used for the identification of chromosomes.
- gammopathy** Plasma protein imbalance caused by markedly increased concentrations of gamma globulin. A monoclonal protein produced by myeloma tumor cells causes most gammopathies.
- Gaucher disease** Rare autosomal recessive disorder of fat metabolism caused by glucocerebrosidase deficiency and characterized by histiocytic hyperplasia in the liver, spleen, lymph nodes, and bone marrow. The characteristic Gaucher cells, which are lipid-filled macrophages whose cytoplasm resembles crumpled tissue paper, are found on the Wright-stained bone marrow aspirate smear.
- Gaussian distribution** Frequency distribution that approximates the distribution of many random variables and is portrayed graphically as a symmetric bell-shaped curve. The peak represents the mean of the distribution and the width of the curve represents the dispersion or variability, which is generally expressed in terms of standard deviation from the mean. Also called a *normal distribution*.
- gene** Segment of a DNA molecule that contains all the information required for synthesis of a protein, including both coding (exon) and noncoding (intron) sequences. Each gene occupies a specific position (locus) on a particular chromosome.
- gene rearrangement** Reorganization of the DNA sequences of a gene. Rearrangement of B-cell and T-cell genes produces an infinite variety of variable-region receptor and immunoglobulin sequences. *Clonal* gene rearrangements occur in lymphoma and lymphocytic leukemia and are detected by flow cytometry to identify these diseases.
- gestational age** Age of the fetus, usually expressed as the time elapsed from the first day of the mother's last menstrual period.
- Glanzmann thrombasthenia (GT)** Severe mucocutaneous bleeding disorder caused by one of a series of mutations in platelet glycoprotein (GP) IIb or IIIa. Normal GP IIb/IIIa recognizes and binds the arginine-glycine-aspartate peptide sequence receptor complex found in fibrinogen and von Willebrand factor. Glanzmann thrombasthenia causes a defect of fibrinogen-dependent platelet aggregation.
- globin** Protein constituent of hemoglobin. Two identical pairs of globin chains bind four heme molecules to form hemoglobin.
- globulins** Class of proteins that are insoluble in water or highly concentrated salt solutions but are soluble in moderately concentrated salt solutions. All plasma proteins are globulins except albumin and prealbumin.
- glossitis** Inflammation of the tongue that causes it to be red and smooth.
- glucose-6-phosphate dehydrogenase (G6PD)** First enzyme of the glucose monophosphate shunt from the Embden-Meyerhoff pathway. G6PD catalyzes the oxidation of glucose-6-phosphate to a lactone, converting the oxidized form of nicotinamide adenine dinucleotide phosphate (NADP) to the reduced form (NADPH).
- glucose-6-phosphate dehydrogenase deficiency** X-linked recessive deficiency of glucose-6-phosphate dehydrogenase characterized by episodes of acute intravascular hemolysis under conditions of oxidative stress, including exposure to oxidative drugs such as quinine.
- glycocalyx** Glycoprotein and polysaccharide covering that surrounds many cells. The platelet glycocalyx is thicker than that of other cells and provides a procoagulant environment.
- glycolysis (Embden-Meyerhof pathway, EMP)** A series of enzymatically catalyzed reactions by which glucose and other sugars are metabolized to yield lactic acid (anaerobic glycolysis) or pyruvic acid (aerobic glycolysis). Metabolism releases energy in the form of adenosine triphosphate.
- glycophorin** Transmembrane red blood cell protein that carries several blood group antigens.
- glycoprotein** Conjugated protein containing one or more covalently linked carbohydrate residues.
- glycoprotein IIb/IIIa (GP IIb/IIIa)** Pair of glycoproteins that function as a receptor for the arginine-glycine-aspartate sequence in fibrinogen and von Willebrand factor. The combination of fibrinogen and glycoprotein IIb/IIIa is essential to platelet aggregation.
- Golgi apparatus** Rigid organelle comprised of numerous flattened sacs and associated vesicles. It is the location of the posttranslational modification and storage of glycoproteins, lipoproteins, membrane-bound proteins, and lysosomal enzymes.
- gout** Painful inflammation caused by excessive plasma uric acid, which becomes deposited as monosodium urate monohydrate in joint capsules and adjacent tendons.
- graft-versus-host disease (GVHD)** Condition including tissue rejection that occurs when immunologically competent cells or their precursors are transplanted into an immunocompromised host who is not histocompatible with the donor. The donor cells engraft and mount an immune response against the host.
- granulocytes** Class of white blood cells in peripheral blood characterized by cytoplasmic granules; includes basophils, eosinophils, and neutrophils.
- granuloma** Nodular, delimited aggregation of inflammatory cells, macrophages, and macrophage-derived multinucleate giant cells surrounded by a rim of lymphocytes and fibroblasts. Granulomas are major sites of cell-mediated immune response to particulate antigens, and they may occur in many organs in chronic granulomatous disease.
- GTG banding (G banding)** In cytogenetic analysis, a procedure in which metaphase chromosomes are treated with trypsin and then stained with Giemsa dye. The areas rich in adenine-thymine, called G+, stain intensely, whereas the areas rich in guanine-cytosine (G-) stain more lightly. The G+ bands correspond with Q bands in Q banding. Banding patterns are used for the identification of chromosomes.
- guaiac test** Test performed on stool emulsions to detect hemoglobin as evidence of gastrointestinal bleeding. Small amounts of blood may be invisible; therefore it is known as a test for occult (hidden) blood. The peroxidase activity of hemoglobin in blood reacts with guaiac to yield a blue color.
- hairy cells** Malignant B lymphocytes seen in the peripheral blood and bone marrow characterized by delicate gray cytoplasm with projections resembling hairs. These cells are seen in hairy cell leukemia.
- haploid** Referring to the number of chromosomes found in sperm or ova, which is only one of each pair of chromosomes found in somatic (diploid) cells. In humans, the haploid number is 23.
- haptoglobin** Plasma protein that irreversibly binds free hemoglobin, forming a complex that is removed by macrophages while conserving iron. Haptoglobin is decreased due to consumption in intravascular hemolysis.
- Heinz bodies** Round blue to purple inclusions attached to inner red blood cell membranes visible when stained with new methylene blue dye. Heinz bodies may be found in multiples and are composed of precipitated hemoglobin in unstable hemoglobin disorders and glucose-6-phosphate dehydrogenase deficiency.
- helmet cell** Helmet-shaped schistocyte that may appear in microangiopathic hemolytic anemia (fragmented red blood cell).
- hemarthroses** Chronic joint bleeds that cause inflammation and immobilization; a symptom of severe hemophilia.
- hematemesis** Vomiting of bright red blood.
- hematocrit (HCT, packed cell volume, PCV)** Proportion of whole blood that consists of red blood cells, expressed as a percentage of the total blood volume.
- hematoidin** Golden yellow, brown, or red crystals that are chemically similar to bilirubin. Hematoidin crystal in a tissue preparation indicates a hemorrhage site.

- hematology** Clinical study of blood cells and blood-forming tissues.
- hematoma** Localized collection of extravasated blood (blood that has escaped from vessel into tissue), usually clotted, in an organ space or tissue, giving the appearance of a bruise.
- hematopathology** Study of the diseases of blood cells and hematopoietic tissue.
- hematopoiesis** Formation and development of blood cells. Hematopoiesis occurs mostly in the bone marrow and peripheral lymphatic tissues.
- hematopoietic microenvironment** Matrix of bone marrow stromal cells and tissue that supports hematopoiesis both structurally and through the production of cytokines and colony-stimulating factors.
- hematopoietic progenitor cell (HPC)** Actively dividing cell that is committed to a single blood cell lineage and is not capable of self-renewal. Therapeutic hematopoietic progenitor cell products intended for transplantation provide both hematopoietic stem cells and progenitor cells.
- hematopoietic stem cell (HSC)** Actively dividing cell that is capable of self-renewal and of differentiation into any blood cell lineage.
- hematuria** Abnormal presence of intact red blood cells in the urine; indicative of kidney or urinary tract disease.
- heme** Pigmented iron-containing nonprotein part of the hemoglobin molecule. There are four heme groups in a hemoglobin molecule, each containing one ferrous ion in the center. Oxygen binds the ferrous ion and is transported from an area of high to low oxygen concentration.
- hemochromatosis** Disease of iron metabolism that is characterized by excess deposition of iron in the tissues. The disease may be inherited or may develop as a complication of a hemolytic anemia, such as β -thalassemia major.
- hemoconcentration** Elevation of the red blood cell, white blood cell, and platelet counts resulting from a decrease in plasma volume, for example, in dehydration.
- hemocytometer (hemacytometer, counting chamber)** Device used to perform visual blood and body fluid cell counts, consisting of a microscopic slide with a depression whose polished glass base is marked with grids and into which a measured volume of a sample of diluted blood is placed and covered with a cover glass. The number of cells in the squares is counted under a microscope and used as a representative sample for calculating the unit volume.
- hemodialysis** Extracorporeal circulation process that substitutes for the kidneys and removes wastes from the blood. Hemodialysis is used to treat patients with renal failure. The patient's blood is shunted from the body through a dialysis machine for diffusion and ultrafiltration and is then returned to the patient's circulation.
- hemoglobin (Hb, HGB)** Tetramer composed of two identical globin chains, each of which binds a heme molecule. Hemoglobin is the primary constituent of red blood cell cytoplasm and transports molecular oxygen from the lungs to the tissues.
- hemoglobin C crystal** Reddish hexagonal cytoplasmic red blood cell crystal described as a "gold bar," or "Washington monument." Typical of homozygous hemoglobin C disease, crystals form as deoxyhemoglobin C polymerizes.
- hemoglobin electrophoresis** Separation and identification of normal and abnormal hemoglobin types based on their relative rates of migration through agarose or polyacrylamide gel in an applied electric field. The rate of migration depends mainly on net charge. Examples of hemoglobin types include Hb A, Hb C, Hb F, and Hb S.
- hemoglobin SC crystal** Irregular reddish cytoplasmic red blood cell crystal described as a "glove" or "pistol." Typical of compound heterozygous hemoglobin SC disease, the crystals form as deoxyhemoglobin S and C polymerize.
- hemoglobinemia** Presence of free hemoglobin in the blood plasma; indicative of intravascular hemolysis.
- hemoglobinopathy** Condition characterized by structural variations in globin genes that result in the formation of abnormal globin chains. Examples are sickle cell anemia and hemoglobin C disease.
- hemoglobinuria** Visible reddish free hemoglobin in the urine; indicative of intravascular hemolysis.
- hemolysis** Disruption of red blood cell membrane integrity that destroys the cell and releases hemoglobin.
- hemolytic anemia** Anemia characterized by a shortened red blood cell (RBC) life span and inability of the bone marrow to adequately compensate by increasing RBC synthesis. Hemolytic anemia may be caused by extrinsic or intrinsic disorders.
- hemolytic disease of the fetus and newborn (HDFN, erythroblastosis fetalis)** Alloimmune anemia caused by maternal IgG antibody that crosses the placenta and binds fetal red blood cell antigens inherited from the father: for instance, maternal anti-A with fetal A antigen. HDFN is characterized by hemolytic anemia, hyperbilirubinemia, and extramedullary erythropoiesis.
- hemolytic uremic syndrome (HUS)** Severe microangiopathic hemolytic anemia that often follows infection of the gastrointestinal tract by *Escherichia coli* serotype O157:H7, which produces an exotoxin. It is characterized by renal failure, thrombocytopenia, the appearance of schistocytes on the peripheral blood film, and severe mucocutaneous hemorrhage.
- hemopexin** Plasma glycoprotein produced in the liver. Hemopexin binds free plasma heme in the absence of haptoglobin.
- hemophilia** Group of hereditary anatomic bleeding disorders caused by a deficiency of a single coagulation factor. The two most common forms are hemophilia A and hemophilia B, deficiencies of factors VIII and IX, respectively.
- hemophilia A (classic hemophilia)** Sex-linked recessive anatomic bleeding disorder caused by a deficiency of coagulation factor VIII.
- hemophilia B (Christmas disease)** Sex-linked recessive anatomic bleeding disorder caused by a deficiency of coagulation factor IX.
- hemophilia C (Rosenthal syndrome)** Autosomal anatomic bleeding disorder caused by a deficiency of coagulation factor XI.
- hemorrhage** Acute severe blood loss requiring intervention and transfusions.
- hemorrhagic disease of the newborn** Neonatal anatomic bleeding caused by vitamin K deficiency.
- hemosiderin** Intracellular storage form of iron found predominantly in liver, spleen, and bone marrow cells. Hemosiderin is a breakdown product of ferritin that appears in iron overload and hemochromatosis. Hemosiderin may be detected microscopically using the Prussian blue iron stain.
- hemosiderinuria** Presence in the urine of hemosiderin, which can be visualized using Prussian blue iron stain; most often an indicator of chronic intravascular hemolysis.
- hemosiderosis** Increased tissue iron stores without associated tissue damage; may progress to hemochromatosis.
- hemostasis** Process by which a series of platelet, endothelial cell, and plasma enzyme systems prevent blood loss through clot formation and maintain blood vessel patency.
- heparin** Naturally occurring mucopolysaccharide anticoagulant classified as a glycosaminoglycan. Heparin is produced by basophils and mast cells. Heparin extracted from porcine mucosa is used as a therapeutic anticoagulant.
- heparin-induced thrombocytopenia with thrombosis (HIT)** Morbid, often fatal effect of unfractionated heparin therapy. Up to 5% of patients receiving unfractionated heparin therapy for more than 5 days develop an antibody to the heparin-platelet factor 4 complex. The antibody and target antigen complex bind platelet Fc receptors and activate platelets, which causes a decrease in platelet count by greater than 30% and venous and arterial thrombosis.
- hepatitis** Inflammation of the liver that damages hepatocytes and releases bilirubin into the plasma.
- hepatitis B virus (HBV)** Causative agent of hepatitis B. The virus is transmitted by contaminated blood or blood products, by sexual contact with an infected person, or by the use of contaminated needles and instruments. Severe infection may cause prolonged illness, destruction of liver cells, cirrhosis, increased risk of liver cancer, or death.

- hepatocyte** Parenchymal liver cell.
- hepatomegaly** Abnormal enlargement of the liver; usually a sign of disease.
- hepatosplenomegaly** Abnormal enlargement of the spleen and liver.
- hereditary elliptocytosis (ovalocytosis)** Hereditary spectrin defect characterized by the presence of elliptocytes in the peripheral blood; often asymptomatic but may be associated with slight anemia.
- hereditary pyropoikilocytosis** Rare hereditary defect of spectrin that causes severe hemolytic anemia beginning in childhood and extreme poikilocytosis with red blood cell morphology resembling that seen in burn patients.
- hereditary spherocytosis** Hereditary defect in a cytoskeletal or transmembrane protein that results in loss of red blood cell membrane and causes hemolytic anemia characterized by numerous spherocytes on the peripheral blood film.
- hereditary stomatocytosis** Hereditary defect of the red blood cell membrane resulting in a complex group of diseases in which the hemolysis is mild to severe and stomatocytes are seen on the peripheral blood film.
- hereditary xerocytosis** Hereditary defect of the red blood cell membrane that results in hemolytic anemia with dehydrated red blood cells and the presence of stomatocytes, target cells, and macrocytes on the peripheral blood film.
- heterochromatin** Portion of DNA that is inactive during transcription to messenger RNA and stains deeply with Wright stain.
- heterophile antibody** Antibody that reacts with an antigen from a species other than that of the antigen that stimulated its production. For example, patients with infectious mononucleosis caused by the Epstein-Barr virus produce an antibody to the virus but also produce heterophile antibodies that react with sheep or horse red blood cells.
- heterozygous** Having two different alleles at corresponding loci on homologous chromosomes. An individual who is heterozygous for a trait has inherited an allele for that trait from one parent and an alternative allele from the other parent. A person who is heterozygous for a genetic disease will manifest the disorder if it is caused by a dominant allele but will remain asymptomatic if the disease is caused by a recessive allele.
- high-molecular-weight kininogen (HMWK, Fitzgerald factor)** Member of the kinin inflammatory system that is digested and activated by kallikrein to form bradykinin. HMWK is one of the *in vitro* contact activators of the coagulation system, which also include prekallikrein and factor XII.
- histiocyte (macrophage)** Mononuclear phagocyte found in all tissues; part of the immune system.
- histochemical analysis** Use of specialized stains to detect enzymes and other chemicals in tissues.
- histocompatibility** Quality or state of immunologic similarity that allows cells or tissues from one individual to be successfully transplanted into another individual. The degree of compatibility is controlled by white blood cell (WBC) surface markers of the human leukocyte antigen (HLA) system of the major histocompatibility complex. Grafts from donors of the immediate family are more compatible than those from unrelated donors.
- histogram** Graph of a frequency distribution. In hematology, a histogram is customarily a line graph generated by an automated analyzer that depicts the frequencies of platelet, white blood cell, or red blood cell volumes in a cell population.
- histology** Science concerned with the microscopic identification of cells and tissues used to identify cellular and tissue disease.
- histone** Nuclear protein that complexes with DNA to form chromatin. Histones provide for DNA folding and condensation and help regulate replication and transcription.
- Hodgkin lymphoma** Solid tumor characterized by painless, progressive hypertrophy of lymphoid tissue, first evident in cervical lymph nodes. Characterized by Reed-Sternberg cells and reactive white blood cells in lymphoid tissue and splenomegaly.
- homocysteine** Naturally occurring sulfur-containing amino acid formed in the metabolism of dietary methionine. Homocysteine concentration in plasma depends upon adequate intake of protein, vitamin B₆, vitamin B₁₂, and folate.
- homocysteinemia** Elevated plasma homocysteine, an independent risk factor for arterial thrombosis, typically caused by vitamin B₆, vitamin B₁₂, or folate deficiency.
- homocysteinuria** Increase or accumulation of homocysteine in urine; indicates an inherited error in an enzyme of the methionine-homocysteine metabolic pathway.
- homozygous** Having two identical alleles at corresponding loci on homologous chromosomes. An individual who is homozygous for a trait has inherited one identical allele for that trait from each parent. A person who is homozygous for a genetic disease caused by a pair of recessive alleles manifests the disorder.
- Howell-Jolly (H-J) bodies** Round blue to purple inclusions in red blood cells (RBCs), usually one per RBC, visible on Wright-stained peripheral blood films. Howell-Jolly bodies are composed of DNA and may indicate severe anemia or splenectomy.
- human leukocyte antigens (HLA, major histocompatibility complex, MHC)** Cell membrane glycoprotein system that forms the basis for antigen presentation in cellular and humoral immunity and enables the immune system to distinguish between self and nonself. HLA class I molecules (HLA-A, -B, -C) are found on most nucleated cells and on platelets. Class II antigens (HLA-DP, -DQ, -DR) are expressed on B lymphocytes, monocytes, macrophages, and dendritic cells.
- humoral immunity** Immune response mediated by B lymphocytes, which produce circulating antibodies (immunoglobulins) in reaction to infectious organisms and other foreign antigens.
- hybridization** In molecular biology, formation of a partially or wholly complementary nucleic acid duplex by association of single strands; used to detect and isolate specific nucleic acid sequences.
- hydrops fetalis** Gross edema of the entire body of a fetus or newborn infant, associated with severe anemia and occurring in hemolytic disease of the fetus and newborn.
- hyperbilirubinemia (icterus, bilirubinemia)** Excess bilirubin in the plasma, which imparts a gold color to the plasma; may indicate hemolytic anemia, liver disease, or bile duct occlusion.
- hypercellular bone marrow** Bone marrow showing an abnormal increase in the concentration of nucleated hematopoietic cells; potentially associated with leukemia or hemolytic anemia.
- hypercoagulability (thrombophilia)** Abnormally increased tendency to develop pathologic thromboses (clots) caused by a number of acquired and congenital factors.
- hyperplasia** Abnormally increased number of cells per unit volume of tissue caused by increased cellular division or abnormal retention. In bone marrow, hyperplasia is evident in hypercellularity. Such hyperplasia may cause enlarged joints or an enlarged forehead, called *frontal bossing*.
- hypersegmented neutrophil** Neutrophil with six or more nuclear lobes or segments; often associated with megaloblastic anemia.
- hypersplenism** Increased hemolytic activity of the spleen caused by splenomegaly, resulting in deficiency of peripheral blood cells and compensatory hypercellularity of the bone marrow.
- hypertension** Persistently elevated blood pressure.
- hyperuricemia** Excess of plasma uric acid or urates; sometimes associated with gout.
- hypocellular bone marrow** Abnormal decrease in the number of nucleated hematopoietic cells present in the bone marrow; may be associated with aplastic anemia or fibrosis.
- hypochromia** Abnormal decrease in the hemoglobin content of red blood cells so that they appear pale with a larger central pallor when stained with Wright stain. These cells are called *hypochromic*.
- hypodiploid** Having fewer than the normal number of somatic cell chromosomes; for example, fewer than 46 chromosomes in humans.

- hypoplasia** Underdevelopment of an organ or tissue, usually resulting from a fewer-than-normal number of cells. A hypoplastic bone marrow is one in which the distribution of nucleated hematopoietic cells is reduced, as in aplastic anemia or fibrosis.
- hypoxia** Diminished availability of oxygen to body tissues, usually secondary to decreased lung capacity or decreased oxygen-carrying capacity of the blood.
- icterus (bilirubinemia, hyperbilirubinemia)** Excess bilirubin in plasma, which imparts a gold color to the plasma; may indicate hemolytic anemia, liver disease, or bile duct occlusion.
- idiopathic** Without a known cause.
- immediate transfusion reaction** Hemolysis that begins within minutes or hours of a blood transfusion and is most commonly caused by an incompatibility of the ABO system.
- immersion oil** Optically clear oil placed in the space between a microscopic specimen and the microscope objective lens. Oil raises the refractive index, improving resolution.
- immune hemolytic anemia** Anemia resulting from shortened red blood cell (RBC) life span caused by antibodies to RBC membrane antigens or complement. The immunoglobulin- or complement-coated RBCs are cleared by splenic macrophages. Anemia results when the bone marrow fails to compensate for RBC consumption.
- immune thrombocytopenic purpura (ITP, idiopathic thrombocytopenic purpura, autoimmune thrombocytopenic purpura, AITP)** Mucocutaneous bleeding secondary to thrombocytopenia caused by a platelet-specific autoantibody that shortens platelet life span. Acute ITP occurs more often in children, while chronic ITP occurs more often in middle-aged adults, more commonly in women than in men.
- immunoblast** Large mitotically active T or B lymphocyte formed as a result of antigenic stimulation.
- immunocompromised** Unable to mount an adequate immune response due to disease or treatment with an immunosuppressive agent. An immunocompromised patient is susceptible to bacterial and viral infections.
- immunocytochemical assays** Laboratory tests in which antibodies labeled with chromophores or fluorophores hybridize specific cellular proteins or nucleic acids to produce a measurable color or fluorescent reaction.
- immunoglobulin (antibody)** Protein of the γ -globulin fraction produced by B lymphocytes and plasma cells that recognizes and binds a specific antigen. Immunoglobulins are the basis of humoral immunity.
- immunophenotyping** Classification of white blood cells and platelets by their membrane antigens. Synthetic antibodies, often monoclonal antibodies produced by hybridoma technology, are used to identify the antigens in flow cytometry.
- immunosuppression** Abnormal state of the immune system characterized by its inability to respond to antigenic stimuli. Immunosuppressive drugs are used to reduce immune responses, particularly in tissue transplant therapy.
- infectious mononucleosis** Acute infection caused by the Epstein-Barr virus, a herpesvirus. Characterized by fever, sore throat, lymphadenopathy, variant lymphocytes, splenomegaly, hepatomegaly, abnormal liver function, and bruising. Laboratory tests used to identify the disease include blood film review for variant lymphocytes, serologic mononucleosis testing, and molecular identification of Epstein-Barr virus.
- integrin** Any of a family of cell-adhesion receptors that mediate interactions between cells and between cells and the extracellular matrix.
- interferon** Natural glycoprotein produced by lymphocytes exposed to a virus or another foreign particle of nucleic acid. Interferon induces the production of translation inhibitory protein (TIP) in noninfected cells. TIP blocks the translation of viral RNA and thus gives other cells protection against the original as well as other viruses.
- interleukin (IL)** Any of a group of compounds that are synthesized by lymphocytes, macrophages, and matrix cells in the marrow and interact with cells to initiate, stimulate, or influence the maturation of blood cells.
- international normalized ratio (INR)** Index computed to normalize prothrombin time (PT) results worldwide. The activity of a PT reagent (thromboplastin) is characterized by manufacturers using the international sensitivity index (ISI), which compares the reagent to the international reference thromboplastin preparation provided by the World Health Organization. Local PTs are adjusted using the following formula: $INR = (PT_{\text{patient}}/PT_{\text{normal}})^{ISI}$, where PT_{patient} is the individual patient's PT and PT_{normal} is the geometric mean of the PT reference interval.
- international reference preparation (IRP)** Human brain-derived thromboplastin maintained by the World Health Organization. Thromboplastin manufacturers worldwide compare their reagents' sensitivity with that of this reference preparation using orthogonal regression to generate an international sensitivity index (ISI) for their products.
- international sensitivity index (ISI)** Index comparing the sensitivity of a given thromboplastin preparation with the sensitivity of the international reference preparation as determined using orthogonal regression. The international sensitivity index is used as an exponent in the equation for calculating the international normalized ratio.
- intracranial hemorrhage (ICH, hemorrhagic stroke)** Bleeding into the brain causing tissue death. Fifteen percent of strokes are caused by intracranial hemorrhage; the remainder are caused by vascular occlusion (ischemia).
- intramedullary hematopoiesis** Formation and development of blood cells within the marrow cavity of a bone.
- intramuscular (IM)** Injected into muscle tissue.
- intravascular hemolysis** Red blood cell destruction that occurs within the blood vessels at a rate exceeding splenic macrophage clearance capacity, releasing hemoglobin into the plasma. Seen in acute hemolytic episodes such as those associated with transfusion reaction, glucose-6-phosphate dehydrogenase deficiency, and sickle cell anemia crisis.
- intrinsic coagulation pathway** Sequence of serine protease reactions leading to fibrin formation, beginning with the in vitro contact activation of factor XII, followed by the sequential activation of factors XI and IX, and resulting in the activation of factor X, which initiates the common pathway of coagulation.
- intrinsic factor (IF)** Glycoprotein secreted by parietal cells of the gastric mucosa that is essential for the intestinal absorption of vitamin B₁₂. A deficiency of, or antibody to, intrinsic factor results in B₁₂ deficiency.
- intron** Nontranslated sequence in a gene. Introns are transcribed to heteronuclear RNA and are excised during the subsequent formation of messenger RNA.
- inversion** Structural chromosome alteration caused by breaks at two locations, reversal of direction of the detached sequence, and reattachment. Inversions have little effect on somatic DNA but cause loss of DNA information in offspring.
- iron deficiency anemia** Microcytic, hypochromic anemia caused by inadequate supplies of the iron needed to synthesize hemoglobin and characterized by pallor, fatigue, and weakness. Often caused by low dietary iron intake or chronic blood loss.
- isoantibody (alloantibody)** Antibody that is produced in response to the presence of foreign antigens; for instance, an antibody to a therapeutic coagulation factor that may render factor therapy ineffective.
- jaundice** Orange-yellow discoloration of the skin, mucous membranes, and sclera. Caused by elevated plasma bilirubin, which signals hepatitis, hemolytic anemia, or common bile duct obstruction.
- karyorrhexis** Nuclear necrosis in which the nucleus ruptures and chromatin disintegrates into formless granules.
- karyotype** Number, form, size, and arrangement of the chromosomes within the nucleus. In cytogenetic laboratory assays mitosis is halted in metaphase and the chromosomes are recorded in a photomicrograph to generate the karyotype.

- kinin** Any of a group of polypeptides that trigger inflammatory activity such as contraction of smooth muscle, vascular permeability, and vasodilation. Examples of kinins are bradykinin and kallidin.
- kininogen** Either of two plasma α_2 -globulins that are kinin precursors, called *high-molecular-weight kininogen* and *low-molecular-weight kininogen*.
- Kleihauer-Betke stain (acid elution slide test)** Test for detecting fetal red blood cells (RBCs) in the maternal circulation. Blood films are immersed in an acid buffer, which causes adult hemoglobin (Hb A) to be eluted from RBCs. The film is stained, and RBCs that have fetal hemoglobin (Hb F) take up the stain.
- Köhler illumination** Light microscope illumination system that consists of a substage light source, field diaphragm, condenser, and aperture diaphragm. These are adjusted in sequence to optimize specimen illumination, resolution, contrast, and depth of field.
- koilonychia** Dystrophy of the fingernails in which they become thin, ridged, and concave. Associated with iron deficiency anemia.
- Kupffer cells** Fixed, highly phagocytic macrophages that line the liver sinusoids. Kupffer cells function like splenic macrophages (littoral cells) to clear senescent red blood cells, immune complexes, and foreign materials.
- lactate dehydrogenase (LD)** Enzyme that catalyzes the reversible conversion of lactate to pyruvate. It is widespread in tissues and is particularly abundant in the kidneys, skeletal muscle, liver, red blood cells, and myocardium. The lactate dehydrogenase assay may be used to detect and monitor cell necrosis in these organs, for instance, acute myocardial infarction or as an indicator of intravascular hemolysis.
- Langerhans cell** Immature dendritic macrophage (histiocyte) with an irregular nucleus and distinctive racquet-shaped cytoplasmic granules called *Birbeck bodies* normally found in the epidermis, oral and vaginal mucosa, and lungs. Langerhans cells have been identified as the cells of origin for a nonproliferating epidermal neoplasm known as *Langerhans cell histiocytosis*.
- leptocyte** Abnormal mature red blood cell that is thin and flat with hemoglobin at the periphery and increased central pallor.
- leukemia** Group of malignant neoplasms of hematopoietic tissues characterized by diffuse replacement of bone marrow or lymph nodes with abnormal proliferating white blood cells and the presence of leukemic cells in the peripheral blood. Leukemia may be chronic or acute and myeloid or lymphoid.
- leukemoid reaction (LR)** Clinical syndrome resembling leukemia in which the white blood cell count is elevated to greater than 50,000/ μ L in response to an allergen, inflammatory disease, infection, poison, hemorrhage, burn, or severe physical stress. Leukemoid reaction usually involves granulocytes and is distinguished from chronic myelogenous leukemia by the use of the leukocyte alkaline phosphatase staining of neutrophils.
- leukocyte** One of the formed elements of the blood. The five families of WBCs are lymphocytes, monocytes, neutrophils, basophils, and eosinophils. WBCs function as phagocytes of bacteria, fungi, and viruses; detoxifiers of toxic proteins that may be produced by allergic reactions and cellular injury; and immune system cells.
- leukocytosis** Abnormally elevated white blood cell count in peripheral blood.
- leukoerythroblastic** Characterized by the presence of immature red blood cells and granulocytes in the peripheral blood and bone marrow.
- leukopenia** Abnormal decrease in the white blood cell count in peripheral blood.
- leukopoiesis** Process by which white blood cells form and develop in the bone marrow and lymph nodes.
- Levey-Jennings chart** Quality control chart used to plot periodic test results for control specimens. The chart indicates the mean and the 1, 2, and 3 standard deviation intervals on both sides of the mean. Deviation from this standard distribution indicates the occurrence of a systematic analytical error.
- ligand** Molecule, ion, or group bound to the central atom of a chemical compound; for example, the oxygen molecule in hemoglobin, which is bound to the central iron atom. Also, a molecule that binds to another molecule; used especially to refer to a small molecule that specifically binds to a larger molecule.
- littoral cells** Fixed, highly phagocytic macrophages that line the sinusoid of the spleen. Littoral cells clear senescent red blood cells, immune complexes, and foreign materials.
- low-molecular-weight heparin (LMWH)** Heparin with an average molecular weight of 5000 to 8000 Daltons produced by enzymatic or chemical digestion of unfractionated heparin. LMWH is used for prophylaxis, and treatment monitoring is required only in conditions of fluid imbalance, such as obesity, renal disease, and pregnancy. LMWH therapy is monitored using the chromogenic anti-factor Xa heparin assay.
- lupus anticoagulant (LA, LAC)** Autoantibody to phospholipid-binding proteins such as β_2 -glycoprotein I, annexin, and prothrombin. May be present as a primary condition or secondary to a collagen disorder such as systemic lupus erythematosus, Sjögren syndrome, or rheumatoid arthritis. Chronic lupus anticoagulant is associated with venous and arterial thrombosis and spontaneous abortion.
- lymphadenopathy** Any disorder characterized by a localized or generalized enlargement of the lymph nodes or lymph vessels.
- lymphoblast** Immature cell found in the bone marrow and lymph nodes, but not normally in the peripheral blood; the most primitive, morphologically recognizable precursor in the lymphocytic series, which develops into the prolymphocyte.
- lymphocytes** Mononuclear, nonphagocytic white blood cells found in the blood, lymph, and lymphoid tissues. Lymphocytes are categorized as B and T lymphocytes and natural killer cells. They are responsible for humoral and cellular immunity and tumor surveillance.
- lymphocytopenia (lymphopenia)** Abnormally reduced lymphocyte count in peripheral blood.
- lymphocytosis** Abnormally increased lymphocyte count in peripheral blood.
- lymphoid** Resembling or pertaining to lymph or tissue and cells of the lymphoid system.
- lymphokines** Biologic response mediators released by both B and T lymphocytes.
- lymphoma** Solid tumor neoplasm of lymphoid tissue categorized as Hodgkin or non-Hodgkin lymphoma and defined by lymphocyte morphology and the histologic features of the lymph nodes.
- lymphopoiesis** Formation and production of lymphocytes, predominantly in the lymph nodes.
- lymphoproliferative** Pertaining to the proliferation of lymphoid cells resulting in abnormally increased lymphocyte counts in peripheral blood, indicating a reactive or neoplastic condition.
- lysosomes** Membrane-bound sacs of varying size distributed randomly in the cytoplasm of granulocytes and platelets. Lysosomes contain hydrolytic enzymes that kill ingested bacteria and digest bacteria and other foreign materials.
- macrocyte** Red blood cell with an abnormally large diameter seen on a peripheral blood film and an elevated mean cell volume. Associated with folate and vitamin B₁₂ deficiency, bone marrow failure, myelodysplastic syndrome, and chronic liver disease.
- macroglobulin** High-molecular-weight plasma globulin, such as α_2 -macroglobulin or an immunoglobulin of the M isotype. Abnormal monoclonal IgM proteins seen in Waldenström macroglobulinemia.
- macrophage (histiocyte)** Mononuclear phagocyte found in all tissues; part of the immune system.
- major histocompatibility complex (MHC, human leukocyte antigen, HLA)** Cell membrane glycoprotein system that forms the basis for antigen presentation in cellular and humoral immunity and enables the immune system to distinguish between self and nonself. MHC class I molecules (HLA-A, -B, -C) are found on most nucleated cells and on platelets. Class II antigens (HLA-DP, -DQ, -DR) are expressed on B lymphocytes, monocytes, macrophages, and dendritic cells.

- malaise** Vague feeling of discomfort and fatigue, often associated with cancer or anemia.
- malaria** Infectious disease caused by one or more of five species of the protozoan genus *Plasmodium*. Malaria is transmitted from human to human by a bite from an infected *Anopheles* mosquito.
- malignant** Describes a cancerous disease that threatens life through its ability to metastasize.
- marker chromosome** In cytogenetic analysis, a chromosome of abnormal size or shape that is an early indicator of neoplastic disease, for instance, the Philadelphia chromosome in chronic myelogenous leukemia.
- mast cell** Connective tissue cell that has large basophilic granules containing heparin, serotonin, bradykinin, and histamine. These substances are released from the mast cell in response to immunoglobulin E stimulation.
- May-Hegglin anomaly** Rare autosomal dominant disorder characterized by thrombocytopenia and granulocytes that contain cytoplasmic inclusions similar to Döhle bodies.
- mean** Value that is derived by dividing the total of a set of values by the number of items in the set; the arithmetic average.
- mean cell hemoglobin (MCH)** Average red blood cell (RBC) hemoglobin mass in picograms computed from the RBC count and hemoglobin level.
- mean cell hemoglobin concentration (MCHC)** Average relative hemoglobin concentration per red blood cell (RBC), expressed in g/dL, computed from the hemoglobin and hematocrit. Relates to Wright-stained RBC color intensity.
- mean cell volume (MCV)** Average red blood cell (RBC) volume in femtoliters computed from the RBC count and hematocrit or directly measured by an automated hematology analyzer. Relates to Wright-stained RBC diameter.
- megakaryoblast** Least differentiated visually identifiable megakaryocyte precursor in a Wright-stained bone marrow aspirate smear. The megakaryoblast cannot be distinguished visually from the myeloblast but is identified using special immunochemical markers.
- megakaryocyte** Largest cell in the bone marrow, measuring 30 to 50 μm and having a multilobed nucleus. Its cytoplasm is composed of platelets, which are released to the blood through the extension of proplatelet processes. Megakaryocytes are identified and enumerated microscopically at low (10 \times) power on a bone marrow aspirate smear.
- megakaryopoiesis (megakaryocytopoiesis)** Production and development of megakaryocytes, the precursors to platelets, in the bone marrow.
- megaloblast** Abnormally large, nucleated, immature precursor of the erythrocytic series; an abnormal counterpart to the pronormoblast. Not only does it have a larger diameter, but the nucleus appears more immature than the cytoplasm. Megaloblasts give rise to macrocytic red blood cells and are associated with megaloblastic anemia, usually caused by folate or vitamin B₁₂ deficiency.
- menorrhagia** Abnormally heavy or prolonged menstrual periods.
- metacentric** Describes a mitotic chromosome having the centromere at the center, with one arm equal in length to the other.
- metamyelocyte** Stage in the development of the granulocyte series, located between the myelocyte stage and the band stage. Characterized by mature, granulated cytoplasm and a bean-shaped nucleus.
- metaphase** Second phase of mitosis in which the chromosomes are aligned at the equatorial plate. Chromosomes at metaphase are maximally contracted and are most easily identified in cytogenetic analysis.
- metaplasia** Conversion of normal tissue cells into another, less differentiated cell type in response to chronic stress or injury. For instance, *myeloid metaplasia* describes hematopoiesis in the spleen or liver.
- metarubricyte (orthochromic normoblast)** Fourth stage of bone marrow erythropoiesis and the last in which the cell retains a nucleus. The nucleus is fully condensed with no parachromatin, and the cytoplasm is 85% hemoglobinized and bluish-pink. When an orthochromic normoblast appears in the peripheral blood, it is called a *nucleated red blood cell*.
- metastasis** Extension or spread of tumor cells to distant parts of the body, usually through the lymphatics or blood vessels.
- methemoglobin** Abnormal form of hemoglobin in which the ferrous ion has become oxidized to the ferric state. Methemoglobin cannot carry oxygen.
- microangiopathic hemolytic anemia (MAHA)** Condition in which narrowing or obstruction of small blood vessels by fibrin or platelet aggregates results in distortion and fragmentation of red blood cells, hemolysis, and anemia. This causes the appearance of schistocytes on a Wright-stained blood film.
- microcyte** Small red blood cell (RBC) with reduced mean cell volume and reduced diameter on Wright-stained peripheral blood film. Microcytes are often associated with iron deficiency anemia and thalassemia.
- microfilament** Intracellular protein that supports the cytoskeleton and assists with cell motility.
- microtubules** Tubulin protein channels that maintain cellular shape, contribute to motility, and make up mitotic spindle fibers and centrioles.
- mitochondria** Round or oval structures distributed randomly in the cytoplasm of a cell. Mitochondria provide the cell's aerobic energy system by producing adenosine triphosphate.
- mitosis** Ordinary process of somatic cell division resulting in the production of two daughter cells that have identical diploid complements of chromosomes.
- monoblast** Most undifferentiated morphologically identifiable precursor of the bone marrow monocytic series; develops into the promonocyte.
- monoclonal** Pertaining to or designating a group of identical cells or organisms derived from a single cell or organism. Also used to describe products from a clone of cells, such as monoclonal antibodies.
- monocyte** Mononuclear phagocytic white blood cell having a round to horseshoe-shaped nucleus with abundant gray-blue cytoplasm filled with fine reddish granules. Circulating precursor to the macrophage, the primary phagocytic cell of most tissues.
- monocytopenia** Abnormally low monocyte count in peripheral blood.
- monocytosis** Abnormally elevated monocyte count in peripheral blood; may indicate infection or a neoplasm.
- mononuclear** Having only one nucleus. Used to describe cells such as monocytes or lymphocytes as distinct from neutrophils, which have nuclei that appear to be multiple and hence are called *segmented* or *polymorphonuclear*.
- Mott cell** Plasma cell containing colorless cytoplasmic inclusions of immunoglobulin called *Russell bodies* that appear similar to vacuoles.
- multiple myeloma (now called plasma cell myeloma)** Malignant neoplasm in which plasma cells proliferate in the bone marrow, destroying bone and resulting in pain, fractures, and excess production of a monoclonal plasma immunoglobulin.
- mutation** Permanent transmissible change in the DNA sequence of a single gene; includes substitution, loss, or gain of one or more nucleotides. Mutations cause the production of abnormal proteins or the loss of a protein.
- myelo-** Prefix relating to the bone marrow or spinal cord and used to identify granulocytic precursors of neutrophils.
- myeloblast** Least differentiated morphologically identifiable bone marrow precursor of the granulocytic series; develops into the promyelocyte. The appearance of myeloblasts in peripheral blood signals acute leukemia.
- myelocyte** Third stage of bone marrow granulocytic series differentiation, intermediate in development between a promyelocyte and a metamyelocyte. In this stage, differentiation of cytoplasmic granules has begun, so myelocytes may be basophilic, eosinophilic, or neutrophilic.

- myelodysplastic syndromes (MDSs)** Group of acquired clonal hematologic disorders characterized by progressive peripheral blood cytopenias that reflect defects in erythroid, myeloid, or megakaryocytic maturation.
- myelofibrosis** Replacement of bone marrow with fibrous connective tissue.
- myeloid** General term used to denote granulocytic cells and their precursors, including basophils, eosinophils, and neutrophils. Lymphoid and erythroid cell lines are excluded, and most morphologists also exclude the monocytic and megakaryocytic cell lines.
- myeloid-to-erythroid (M:E) ratio** Proportion of myeloid cells to nucleated erythroid precursors in bone marrow aspirate. The myeloid-to-erythroid ratio is used to evaluate hematologic cell production. Excluded from the myeloid cell count are monocytic and lymphoid precursors and plasma cells.
- myelokathexis** Inherited severe neutropenia and lymphopenia characterized by hypersegmented neutrophils and myeloid hyperplasia. Part of WHIM syndrome (*warts, hypogammaglobulinemia, infections, and myelokathexis*).
- myeloperoxidase (MPO)** Enzyme that occurs in primary granules of promyelocytes, myelocytes, and neutrophils and exhibits bactericidal, fungicidal, and virucidal properties. Cytochemical stains that detect myeloperoxidase are used to identify myeloid precursors in acute leukemia.
- myeloproliferative neoplasms (MPN, myeloproliferative disorders, MPD)** Group of neoplasms characterized by proliferation of myeloid tissue and elevations in one or more myeloid cell types in the peripheral blood. Myeloproliferative neoplasms include myelofibrosis with myeloid metaplasia, essential thrombocythemia, polycythemia vera, and chronic myelogenous leukemia.
- myoglobin** Monomeric heme-containing protein in muscle. Combines with oxygen released by red blood cells, stores it, and transports it to the mitochondria of muscle cells, where it generates energy.
- necrosis** Localized tissue death that occurs in groups of cells in response to disease or injury.
- neonatal** Pertaining to the first 28 days after birth.
- neoplasm** Any abnormal growth of new tissue; can be malignant or benign. The term is usually applied to cancerous cells.
- neuropathy** Any disorder affecting the nervous system.
- neutropenia** Abnormally reduced neutrophil count in peripheral blood. Often associated with chemotherapy, it exposes the patient to the risk of infection.
- neutrophil** Mature segmented (polymorphonuclear) white blood cell with fine pink-staining cytoplasmic granules in a Wright-stained peripheral blood film. Neutrophils ingest bacteria and cellular debris.
- neutrophilia** Abnormally elevated neutrophil count in peripheral blood; often indicates bacterial infection and may be associated with chronic myelogenous leukemia.
- nondisjunction** Faulty distribution of chromosomal elements during mitosis or meiosis.
- non-Hodgkin lymphoma (NHL)** Solid tumors of lymphoid tissue classified by histologic features and lymphocytic morphology. Should be distinguished from Hodgkin lymphoma, which is caused by proliferation of Reed-Sternberg cells and accumulation of reactive peripheral blood cells.
- nonsteroidal antiinflammatory drugs (NSAIDs)** Antiinflammatory, analgesic, and antiplatelet drugs, other than steroids. NSAIDs include aspirin, which acetylates cyclooxygenase and reduces platelet activation, as well as naproxen, acetaminophen, and ibuprofen.
- normochromic** Describes a Wright-stained red blood cell with normal color and normal hemoglobin content with a mean cell hemoglobin concentration within the reference interval.
- normocyte** Normal, mature red blood cell with a mean cell volume within the reference interval.
- nucleated red blood cell (NRBC)** Red blood cell (RBC) in peripheral blood that possesses a nucleus; often an orthochromic normoblast (metarubricyte). Nucleated RBCs falsely raise manual white blood cell (WBC) counts, which requires a manual WBC count correction.
- nucleolus** Round or irregular pocket of messenger and ribosomal RNA in the nucleus. Nucleoli are observed by morphologists and are used to distinguish cell differentiation stages.
- nucleoside** Glycoside of purine and pyrimidine bases. In RNA and DNA, the glycosides are the pentoses ribose and deoxyribose.
- nucleotide** Phosphoric ester of a nucleoside. Allows for the formation of phosphodiesterase bridges between nucleotides to form RNA and DNA.
- nucleus** Cellular organelle containing DNA and RNA. Stores genetic information and controls cell functions.
- nucleus-to-cytoplasm (N:C) ratio** Estimated volume of a Wright-stained nucleus in comparison to the volume of the cytoplasm. The nucleus-to-cytoplasm ratio is used to differentiate cell developmental stages.
- numerical aperture (NA)** Number stamped on the barrel of a microscope lens designating the quality of the microscope objective. The higher the number, the greater the lens's resolution.
- objective** Microscope lens closest to the specimen. Most clinical grade microscopes provide 10× dry, 50× oil immersion, and 100× oil immersion objectives.
- ocular** Microscope lens, usually 10×, nearest the eye.
- oncogene** Gene capable, under certain conditions, of causing the initial and continuing conversion of normal cells into cancer cells. A mutated proto-oncogene.
- opsonization** Process by which an antibody or complement attaches itself to foreign material (bacteria), triggering or enhancing phagocytosis by white blood cells.
- optical scatter** Scattering of light caused by the interaction of absorption, diffraction, refraction, and reflection. Hematology analyzers that use both laser and nonlaser light apply the principle of light scatter to perform cell counting and identification. The angle of light scatter correlates with various cell features such as volume, density, and cellular complexity.
- orthochromic normoblast (metarubricyte)** Fourth stage of bone marrow erythropoiesis and the last in which the cell retains a nucleus. The nucleus is fully condensed with no parachromatin; the cytoplasm is 85% hemoglobinized and bluish-pink. When an orthochromic normoblast appears in the peripheral blood, it is called a *nucleated red blood cell*.
- osmotic fragility test** Assay in which whole blood is pipetted to each of a series of saline solutions of graduated concentration. The series begins with water and increases in concentration to normal (0.85%) saline. The osmotic fragility test is used to detect spherocytosis, because spherocytes rupture in saline concentrations near the normal level. It may also detect target cells, which, owing to their reduced hemoglobin content, are able to withstand osmotic stress and rupture only at very dilute saline concentrations.
- osteoblast** Bone-forming cell.
- osteoclast** Large multinuclear cell associated with the absorption and removal of bone. May be confused with a megakaryocyte.
- oval macrocyte** Oval red blood cell with an increased diameter seen in peripheral blood. Characteristic of megaloblastic anemia.
- ovalocyte (elliptocyte)** Oval red blood cell seen in peripheral blood in the membrane disorder hereditary elliptocytosis. May be found in low numbers in healthy states and in other anemias such as iron deficiency and thalassemia major.
- oxygen affinity** Ability of hemoglobin to bind oxygen molecules.
- oxygen dissociation curve** Graphic expression of the affinity between oxygen and hemoglobin or the concentration of oxygen bound at equilibrium to the hemoglobin in blood as a function of oxygen pressure.
- oxyhemoglobin** Hemoglobin that contains bound oxygen.
- P arm** Smaller (petite) arm of a chromosome.
- packed cell volume (PCV, hematocrit, HCT)** Proportion of whole blood that consists of red blood cells, expressed as a percentage of the total blood volume.

- pallor** Unnatural paleness or absence of color from the skin.
- pancytopenia** Marked reduction in the count of red blood cells, white blood cells, and platelets in peripheral blood.
- Pappenheimer bodies (siderotic granules)** Red blood cell inclusions composed of ferric iron. On Prussian blue iron stain preparations, they appear as multiple dark blue irregular granules. On Wright stain preparations they appear as pale blue clusters.
- parachromatin** Pale-staining portion of the nucleus, roughly equivalent to euchromatin.
- parenteral** Pertaining to administration of drugs or other compounds by means other than oral administration; includes intramuscular and intravenous administration.
- paroxysmal cold hemoglobinuria (PCH)** Rare acquired autoimmune hemolytic anemia in which the Donath-Landsteiner autoantibody binds red blood cells during exposure to cold, producing acute hemolysis and hematuria upon warming.
- paroxysmal nocturnal hemoglobinuria (PNH)** Acquired hemolytic anemia due to a stem cell clonal mutation that causes the cell to lack glycosylphosphatidylinositol-anchored proteins, including CD55 and CD59, two proteins that normally protect the red blood cell (RBC) from complement activation and hemolysis. RBCs therefore have an increased susceptibility to complement, which results in intravascular hemolysis and hemoglobinuria that occur in irregular episodes.
- partial thromboplastin time (PTT, activated partial thromboplastin time, APTT)** Clot-based screening test for intrinsic coagulation, which is prolonged in deficiencies of prekallikrein, high-molecular-weight kininogen, and factors XII, XI, IX, VIII, X, V, II, and fibrinogen. Calcium chloride, phospholipid, and activator are added to patient plasma. The interval from the addition of reagent to clot formation is recorded. The test is used to monitor unfractionated heparin therapy, to screen for intrinsic pathway deficiencies, and to screen for lupus anticoagulant.
- pathogenesis** Chemical and biologic events in cells and tissue by which disease occurs and progresses.
- pathognomonic** Specifically characteristic of a given disease; indicating a sign or symptom from which a diagnosis can be made.
- Pelger-Huët anomaly** Autosomal dominant asymptomatic anomaly of neutrophil nuclei, which fail to segment and appear dumbbell shaped or peanut shaped ("pince-nez" nuclei). Pelgeroid nuclei are more common and resemble the nuclei of Pelger-Huët anomaly, but may indicate myelodysplasia or may appear during chemotherapy.
- percutaneous coronary intervention (PCI)** Any catheter-based technique for the management of coronary artery occlusion, such as angiography, angioplasty, stent placement within a coronary artery, or cardiac catheterization.
- peripheral artery occlusion (PAO)** Blockage of a noncardiovascular, noncerebral artery—for example, a carotid or brachial artery—by thrombus formation.
- pernicious anemia** Progressive autoimmune disorder that results in megaloblastic macrocytic anemia due to a lack of, or antibodies to, parietal cells or intrinsic factor essential for the absorption of vitamin B₁₂.
- personal protective equipment (PPE)** Clothing that is used to prevent blood or other potentially infectious biologic substances from contacting clothing, eyes, mouth, or mucous membranes. Includes, for example, gloves, fluid-impermeable laboratory coats, and eye protection.
- petechiae** Pinpoint purple or red spots on the skin or mucous membranes, approximately 1 mm in diameter, indicating small bleeds within the dermal or submucosal layers. May indicate a systemic bleeding disorder.
- phagocyte** Cell that is able to surround, engulf, and digest microorganisms and cellular debris. Macrophages and neutrophils are phagocytes.
- phagocytosis** Ingestion of large particles or live microorganisms into a cell.
- phagosome** Membrane-bound cytoplasmic vesicle in a phagocyte containing the phagocytosed material.
- Philadelphia chromosome** Reciprocal translocation of the long arm of chromosome 22 to chromosome 9; definitive for the diagnosis of chronic myelogenous leukemia. The mutation results in the fusion of the *BCR* and *ABL* genes and abnormal tyrosine kinase production.
- phlebotomy** Use of a needle to puncture a vein and collect blood.
- phytohemagglutinin (PHA)** Lectin extracted from the red kidney bean that causes red blood cell agglutination by binding to *N*-acetyl- β -glucosamine. Also a mitogen that induces T-lymphocyte proliferation in culture.
- pinocytosis** Cellular ingestion of small particles or liquids.
- pitting** Removal by the spleen of material from within red blood cells (RBCs) without damage to the RBCs; for example, removal of nuclei or Howell-Jolly bodies.
- plasma** Fluid portion of the blood in which the formed elements (white blood cells, red blood cells, and platelets) are suspended.
- plasma cell** Fully differentiated B lymphocyte found in the bone marrow and lymphoid tissue, and occasionally in peripheral blood. It contains an eccentric nucleus with deeply staining chromatin and abundant dark blue cytoplasm. The Golgi apparatus produces a perinuclear halo due to its high lipid content. Plasma cells secrete antibody in the humoral immune response.
- plasma frozen within 24 hours (FP24)** Plasma that is separated from whole-blood donations and frozen within 24 hours of collection. FP24 contains all of the plasma procoagulants and control proteins, including adequate levels of the labile factors V and VIII. FP24 is used for replacement therapy in acquired multiple-factor deficiencies or in single-factor deficiencies when factor concentrates are not available.
- plasmin** Active form of plasminogen. Plasmin binds fibrin and, when activated by tissue plasminogen activator, digests fibrin to form fibrin degradation products in the fibrinolytic process.
- plasminogen** Inactive (zymogen) plasma precursor of plasmin.
- plasminogen activator** Substance that cleaves plasminogen and converts it into plasmin; includes urokinase, which is secreted by renal endothelial cells, and tissue plasminogen activator, which is secreted by all endothelia. Synthetic plasminogen activators are used therapeutically to clear coronary artery clots after acute myocardial infarction.
- plasminogen activator inhibitor 1 (PAI-1)** Endothelial cell inhibitor of tissue plasminogen activator; controls fibrinolysis.
- platelet (thrombocyte)** Smallest of the formed elements in blood; disk-shaped, 2 to 4 μ m in diameter, nonnucleated cell formed in the bone marrow from the cytoplasm of megakaryocytes. Platelets trigger and control blood coagulation.
- platelet adhesion** Platelet attachment to subendothelial collagen, part of a sequential mechanism leading to the initiation and formation of a thrombus or hemostatic plug. Platelet adhesion requires von Willebrand factor and platelet receptor glycoprotein Ib/IX/V.
- platelet aggregation** Platelet-to-platelet binding, part of a sequential mechanism leading to the initiation and formation of a thrombus or hemostatic plug. Requires fibrinogen and platelet membrane receptor glycoprotein IIb/IIIa.
- platelet factor 4 (PF4)** Protein released from platelet α -granules that binds and inhibits heparin. The heparin-PF4 complex is implicated in heparin-induced thrombocytopenia with thrombosis.
- platelet-poor plasma (PPP)** Plasma centrifuged to achieve a platelet count of less than 10,000/ μ L. PPP is required for all coagulation testing, and only PPP may be frozen.
- platelet-rich plasma (PRP)** Plasma centrifuged at 50 \times g to achieve a platelet count of 200,000/ μ L to 300,000/ μ L. Platelet-rich plasma is used for light-transmission platelet aggregometry.
- platelet satellitosis (satellitism)** Antibody-mediated in vitro adhesion of platelets to segmented neutrophils. Occurs primarily in specimens anticoagulated with ethylenediaminetetraacetic acid (EDTA) and causes pseudothrombocytopenia.

- pleomorphic** Occurring in various distinct forms; having the ability to exist in various forms and to change from one form to another.
- pluripotential stem cell** Stem cell that has the potential to differentiate into one of several types of hematopoietic progenitor cells, including lymphocytic, monocytic, granulocytic, megakaryocytic, and erythrocytic lineages and nonhematopoietic cells.
- pneumatic tube system** System of tubes for transporting blood specimens or other small materials in hospitals by forced air.
- poikilocytosis** Presence in the peripheral blood of red blood cells with varying or bizarre shapes.
- point-of-care (POC) testing** Rapid-turnaround clinical tests performed outside of the clinical laboratory at or near the patient; usually performed by nonlaboratory personnel but managed for quality by laboratory personnel.
- polychromatic (polychromatophilic)** Having a staining quality in which both acid and basic stains are incorporated. Usually used to denote a mixture of pink and blue in the cytoplasm of Wright-stained cells.
- polychromatic normoblast (polychromatophilic normoblast, rubricyte)** Precursor in the erythrocytic maturation series, intermediate between the basophilic normoblast (prorubricyte) and the orthochromic normoblast (metarubricyte). In this stage, differentiation is based on the decreasing cell diameter and the gray-blue cytoplasm as hemoglobin first becomes visible.
- polychromatic or polychromatophilic red blood cell (reticulocyte)** Immature but anucleate red blood cell (RBC) with bluish-pink cytoplasm on a Wright-stained blood film. When new methylene blue dye is used, the cytoplasm of these cells has a meshlike pattern of dark blue threads and particles, vestiges of the endoplasmic reticulum. Reticulocytosis or polychromatophilia indicates bone marrow regeneration activity in hemolytic anemia or acute blood loss.
- polychromatophilia (reticulocytosis)** Elevated reticulocyte count on a peripheral blood film stained with new methylene blue dye or increase in the number of polychromatophilic red blood cells on a Wright-stained blood film. Reticulocytosis or polychromatophilia indicates bone marrow regeneration activity in hemolytic anemia or acute blood loss.
- polyclonal** Describes a group of cells or organisms derived from several cells. Each cell is identical to its parent cells (i.e., a clone), but since all cells of the group did not derive from the same cell, the group of cells is mixed. A polyclonal antibody is developed within a laboratory animal and not a hybridoma.
- polycythemia (erythrocytosis)** Elevated red blood cell count, hemoglobin, and hematocrit in peripheral blood, usually in response to chronic hypoxia.
- polycythemia vera (PV)** Myeloproliferative neoplasm in which a somatic mutation leads to a marked increase in the red blood cell (RBC) count, hematocrit, hemoglobin, white blood cell count, platelet count, and red blood cell mass. RBC precursors are hypersensitive to erythropoietin.
- polymerase chain reaction (PCR)** Laboratory process in which a strand of DNA is replicated in a thermocycler to produce millions of copies within a few hours.
- polymorphonuclear neutrophil (PMN, segmented neutrophil, seg)** White blood cell whose nucleus is condensed into two to five segments or lobes connected by filaments. Distinguished from mononuclear cells such as monocytes and lymphocytes.
- polypliod** Having more than the characteristic diploid set of chromosomes; for example, triploid (3×) or tetraploid (4×).
- porphyria** Hereditary anemia caused by impaired heme synthesis with the accumulation of porphyrin and its precursors. Acquired porphyria may be seen in acute lead poisoning.
- porphyrin** Product of the metabolism of a group of iron- or magnesium-free pyrrole derivatives such as protoporphyrin and protoporphyrinogen that incorporates ferrous iron to form heme.
- posttransfusion purpura** Antibody-induced thrombocytopenia in patients who have received multiple transfusions of red blood cells or platelet concentrate. Antibodies to donor platelets cross-react with patient platelets to cause potentially life-threatening thrombocytopenia.
- postmenstrual age** Time elapsed between the first day of the mother's last menstrual period and birth (gestational age) plus the time elapsed after birth (chronologic age). For example, a preterm infant born at a gestational age of 32 weeks who is currently 10 weeks old (chronologic age) would have a postmenstrual age of 42 weeks.
- precision** Degree to which the results of replicate analyses of a sample parallel each other, often expressed as coefficient of variation or percent coefficient of variation.
- precursor** Differentiating (immature) hematopoietic cell stage that is morphologically identifiable as belonging to a given cell line; for example, pronormoblasts (rubriblasts) are precursors of basophilic normoblasts (prorubricytes) in erythropoiesis. *Precursor* may also refer to the inactive zymogen forms of coagulation factors; for example, prothrombin is a precursor of thrombin.
- preeclampsia** Pathologic condition of late pregnancy characterized by edema, proteinuria, and hypertension; may lead to eclampsia, which presents with seizures and is often fatal.
- prekallikrein (PK, pre-K, Fletcher factor)** Member of the kinin inflammatory system that forms active kallikrein upon digestion by kininogen. Pre-K helps to trigger in vitro coagulation contact activation. Pre-K deficiency prolongs the partial thromboplastin time but has no clinical consequence.
- primary hemostasis** First phase of hemostasis in which the blood vessels contract to seal the wound and platelets and von Willebrand factor fill the open space by forming a platelet plug.
- primary standard** Reference material that is of fixed and known composition and is capable of being prepared in essentially pure form.
- primer** Short piece of synthetic DNA complementary to a target DNA sequence. The primer acts as a point from which replication can proceed, as in a polymerase chain reaction.
- procoagulant** Coagulation (clotting) factor. During the coagulation process, inactive procoagulants become activated to form serine proteases or cofactors and function together to produce a localized thrombus.
- progenitor** Undifferentiated (immature) hematopoietic cell that is committed to a cell line but cannot be identified morphologically.
- polymphocyte** Developmental form in the lymphocytic series that is intermediate between the lymphoblast and the lymphocyte.
- promegakaryocyte** Morphologically identifiable bone marrow cell stage that is intermediate between the megakaryoblast and the megakaryocyte.
- promonocyte** Precursor in the monocytic series; the cell stage intermediate in development between the monoblast and the monocyte.
- promyelocyte** Precursor in the granulocytic (myelocytic) series that is intermediate in development between a myeloblast and a myelocyte; contains primary granules.
- pronormoblast (rubriblast)** Undifferentiated (immature) hematopoietic cell that is the most primitive morphologically identifiable precursor in the erythrocytic series; differentiates into the basophilic normoblast (prorubricyte).
- prorubricyte (basophilic normoblast)** Second identifiable stage in bone marrow erythrocytic maturation; it is derived from the pronormoblast (rubriblast). Typically 10 to 15 μm in diameter, the prorubricyte has cytoplasm that stains dark blue with Wright stain.
- prostaglandin (PG)** Any of a family of unsaturated 20-carbon fatty acids that are cleaved from cell membrane phospholipids and serve as intracellular activators and inhibitors. The prostaglandins include thromboxane A₂, a platelet activator, and prostacyclin, a platelet inhibitor produced by endothelial cells.
- protein C (PC)** Vitamin K-dependent coagulation control protein. A plasma serine protease activated by thrombin-thrombomodulin and stabilized by its cofactor, protein S, protein C inhibits coagulation by inactivating factors Va and VIIIa.
- protein S (PS)** Vitamin K-dependent coagulation control protein. Serves as a stabilizing cofactor for protein C, enabling activated protein C, a serine protease, to inactivate factors Va and VIIIa.

- proteins in vitamin K antagonism (PIVKA)** Name given to the forms of factors II, VII, IX, and X and proteins C, S, and Z that, under conditions of vitamin K absence or antagonism, lack a second glutamic acid carboxyl group as required for binding to Ca^{2+} and phospholipid. Also called *des- γ -carboxyl coagulation factors*. The des- γ -carboxyl forms of these factors cannot participate in coagulation reactions. Vitamin K antagonism is the basis for oral anticoagulant therapy with warfarin (Coumadin).
- proteolytic** Pertaining to any substance that digests protein by hydrolyzing primary peptide bonds.
- prothrombin (coagulation factor II)** Plasma precursor of the coagulation factor thrombin. It is converted to thrombin by activated factor X complexed to factor V.
- prothrombin time (protime, PT)** Test to measure the activity of coagulation factors I (fibrinogen), II (prothrombin), V, VII, and X, which participate in the extrinsic and common pathways of coagulation. Thromboplastin and calcium are added to plasma and the clotting time is recorded. Widely used to monitor warfarin therapy.
- proto-oncogene** Normal gene controlling the cell cycle and other essential cell functions that, upon activation, may become an oncogene.
- pseudo-Pelger-Huët cell (Pelgeroid cell)** Hyposegmented, hypogranular neutrophils that resemble Pelger-Huët cells. Helpful in the diagnosis of leukemia, myeloproliferative neoplasms, and myelodysplastic syndromes.
- pseudoleukocytosis** Falsely increased white blood cell (WBC) count indicating mobilization of the marginal WBC pool caused by strenuous physical activity, cold, or fever. Also a falsely elevated WBC count due to interferences such as platelet clumping or lysis-resistant red blood cells.
- pseudothrombocytopenia** Falsely decreased platelet count often caused by platelet satellitosis.
- pulmonary embolism (PE)** Pathologic movement of a proximal fragment of clot from a deep vein thrombosis through the right side of the heart to the pulmonary circulation, where it lodges in an artery and causes infarction of the lung. Approximately one third of pulmonary emboli are fatal within 1 hour.
- purpura** Purple skin discoloration, typically rounded with a diameter greater than 3 mm, seen in mucocutaneous bleeding. Usually seen in thrombocytopenia, platelet disorders, von Willebrand disease, or vascular disorders such as scurvy.
- pyknosis** Degeneration of a cell in which the nucleus shrinks in size and the chromatin condenses to a solid, structureless mass or masses. Part of the process of apoptosis, or indicative of the effects of chemotherapy.
- pyropoikilocytosis (hereditary pyropoikilocytosis)** Rare hereditary defect of spectrin that causes severe hemolytic anemia beginning in childhood with extreme poikilocytosis in which the red blood cell morphology resembles that seen in burn patients.
- pyruvate kinase (PK)** Enzyme that converts phosphoenolpyruvate to pyruvate generating two molecules of ATP; essential for aerobic and anaerobic glycolysis.
- pyruvate kinase deficiency** Autosomal recessive disorder resulting in a deficiency of pyruvate kinase, the enzyme that converts phosphoenolpyruvate to pyruvate; causes hemolytic anemia by reducing red blood cell life span. It is the most common enzyme deficiency of the Embden-Meyerhof pathway.
- Q arm** Long arm of a chromosome.
- Q banding (quinacrine banding)** In cytogenetic analysis, a procedure in which metaphase chromosomes are stained with fluorescent quinacrine dye. The areas rich in adenine-thymine, called Q+, fluoresce intensely, whereas the areas rich in guanine-cytosine (Q-) fluoresce more lightly. The Q+ bands correspond with G bands in Giemsa stain-based G banding. Banding patterns are used to identify chromosomes.
- qualitative analysis** Study of a sample to determine the presence, but not the concentration, of specific chemicals.
- quality control** Term used to refer to the control and monitoring of the testing process to ensure that the results are valid and reproducible.
- quantitative analysis** Determination of the concentration of an analyte.
- radiotherapy** Treatment of neoplastic disease using x-rays or gamma rays to deter the proliferation of malignant cells by disrupting mitosis or impairing DNA synthesis.
- Raynaud phenomenon (acrocyanosis)** Persistent symmetrical cyanosis (blotchy blue or red discoloration) of the skin of the digits, palm, wrists, and ankles upon prolonged exposure to cold.
- reactive lymphocytes (atypical, transformed, or variant lymphocytes)** Lymphocytes whose altered morphology includes stormy blue cytoplasm and lobular or irregular nuclei. Variant lymphocytes indicate stimulation by a virus, particularly Epstein-Barr virus, which causes infectious mononucleosis.
- recessive** Denoting an allele whose product or effect is masked by a dominant allele at the corresponding locus. When an individual has two recessive genes, he or she is homozygous recessive, and the trait is expressed.
- red blood cell (RBC) indices** Numerical representations of average RBC volume (mean cell volume), hemoglobin mass (mean cell hemoglobin), and relative hemoglobin concentration (mean cell hemoglobin concentration). Indices are computed from the RBC count, hemoglobin, and hematocrit values. The mean cell volume is directly measured by some hematology analyzers.
- red cell distribution width (RDW)** Coefficient of variation of red blood cell volume. An increased red cell distribution width indicates anisocytosis.
- red marrow** Hematopoietic bone marrow, in contrast to yellow, fatty bone marrow.
- Reed-Sternberg cell** Giant, typically binucleate cell whose halves are mirror images. The nuclei are enclosed in abundant cytoplasm and contain prominent nucleoli. The presence of Reed-Sternberg cells is the definitive histologic characteristic of Hodgkin disease.
- reference interval (RI)** Range of test results for a given analyte that is seen in a healthy population of individuals, typically computed as the mean plus or minus two times the standard deviation. Each laboratory must define the reference interval for the instrument that is being used and for the population that is being served.
- refractive index** Speed at which light travels in air, divided by the speed at which light travels through another medium, such as immersion oil. The refractive index of oil is similar to the refractive index of glass.
- reliability** Extent to which a method is able to maintain both accuracy and precision over a defined period of time.
- remission** Partial or complete disappearance of the clinical and laboratory characteristics of a chronic or malignant disease.
- replication** DNA duplication or synthesis prior to mitosis; may also be used to refer to mitosis.
- reptilase** Thrombin-like enzyme isolated from the venom of *Bothrops atrox* that catalyzes the conversion of fibrinogen to fibrin in a manner similar to thrombin.
- reshathing device** Device that allows safe one-handed recapping of a blood collection needle to prevent needle stick injury.
- resolution** In microscopy, the smallest distance between which two adjacent objects can be distinguished. A measure of image and lens quality that relates to the smallest feature that can be seen with a set of lenses.
- restriction endonuclease** Enzyme that cleaves DNA at a specific nucleotide site.
- reticulocyte (polychromatic or polychromatophilic red blood cell)** Immature but anucleate red blood cell (RBC) that shows a meshlike pattern of dark blue threads and particles, vestiges of the endoplasmic reticulum, when stained with new methylene blue vital dye. In a Wright-stained blood film, no filaments are seen but the cytoplasm stains bluish-pink and the cell is called a *polychromatic* or *polychromatophilic RBC*. Reticulocytosis or polychromatophilia indicates bone marrow regeneration activity in hemolytic anemia or acute blood loss.
- reticulocyte production index (RPI)** Index calculated to correct for the presence of shift reticulocytes that otherwise may falsely elevate the visual reticulocyte count.

- reticulocytosis (polychromatophilia)** Elevated reticulocyte count on a peripheral blood film stained with new methylene blue dye or increase in the number of polychromatophilic red blood cells on a Wright-stained blood film. Reticulocytosis or polychromatophilia indicates bone marrow regeneration activity in hemolytic anemia or acute blood loss.
- reticuloendothelial system (RES)** System of fixed and motile phagocytic macrophages and their precursor monocytes engaged in primary efferent immunity. Macrophages are found in every organ and tissue.
- retrovirus** Any of a family of RNA viruses containing the enzyme reverse transcriptase.
- reverse transcription polymerase chain reaction (RT-PCR)** Polymerase chain reaction amplification process that produces complementary DNA from the messenger RNA present in an RNA sample extracted from patient cells.
- Rh immune globulin (RhIg)** Solution containing antibodies specific for the Rh(D) antigen given intramuscularly to an Rh(D)-negative mother with an Rh(D)-positive fetus or infant to prevent sensitization of the mother to the infant's D antigen. It is given antepartum at 28 weeks' gestation and again within 72 hours of delivery.
- Rh-null disease** Hemolytic anemia in persons who lack all Rh antigens (Rh null); marked by spherocytosis, stomatocytosis, and increased osmotic fragility.
- ribonucleic acid (RNA)** Single strand of polynucleotides connected by ribose molecules. RNA base sequences are transcribed from DNA and are the basis for translation to proteins. Major types of RNA include messenger RNA, ribosomal RNA, and transfer RNA.
- ribosomes** Granules embedded in the membranes of endoplasmic reticulum that are composed of protein and RNA. Ribosomes are the sites for primary protein translation from messenger and transfer RNA.
- ring sideroblast** Nucleated red blood cell precursor with at least five iron granules that circle at least one third of the nucleus. These cells, visible with Prussian blue stain, are the pathognomonic finding in refractory anemia with ring sideroblasts.
- ristocetin** Antibiotic no longer in clinical use that facilitates the in vitro interaction of von Willebrand factor with platelet membrane glycoprotein Ib/IX/V. Used as an agonist in platelet aggregation to test for von Willebrand disease.
- Romanowsky stain** Prototype of the many eosin-methylene blue stains for blood cells and malarial parasites, including Wright and Giemsa stain.
- rouleaux** Aggregation of stacked red blood cells caused by elevated plasma proteins and abnormal monoclonal proteins.
- rubriblast (pronormoblast)** Undifferentiated (immature) hematopoietic cell that is the most primitive morphologically identifiable precursor in the erythrocytic series; differentiates into the basophilic normoblast (prorubricyte).
- rubricyte (polychromatic or polychromatophilic normoblast)** Precursor in the erythrocytic maturation series that is intermediate between the basophilic normoblast (prorubricyte) and the orthochromic normoblast (metarubricyte). In this stage, differentiation is based on the decreasing cell diameter and the gray-blue cytoplasm as hemoglobin first becomes visible.
- Russell bodies** Plasma cell cytoplasmic inclusions that appear similar to vacuoles containing aggregates of immunoglobulins. Plasma cells with Russell bodies are called *Mott cells*.
- Russell viper venom time** Rarely used coagulation assay similar to the prothrombin time. Russell viper venom activates the coagulation factor common pathway at the level of factor X. The dilute Russell viper venom time assay is used routinely in lupus anticoagulant screening.
- scatterplot** Plot on rectangular coordinates of paired observations of two random variables, with each observation plotted as one point on the graph; the scatter or clustering of points provides an indication of the relationship between the two variables. In hematology a typical scatterplot graphs cell volume against cytoplasmic complexity.
- schistocyte (schizocyte)** Fragmented red blood cell characteristic of microangiopathic hemolytic anemia, severe burns, disseminated intravascular coagulation, and prosthetic mechanical trauma.
- scurvy** Deficiency of vitamin C (ascorbic acid) causing connective tissue breakdown. Marked by weakness, anemia, spongy gums, and a tendency to mucocutaneous bleeding.
- secondary hemostasis** Second phase of hemostasis involving the activation of plasma coagulation proteins to produce a fibrin clot.
- secondary standard (calibrator)** Calibration material for which the analyte concentration has been ascertained by reference to a primary standard or by controlled reference assays.
- segmented neutrophil (seg, polymorphonuclear neutrophil, PMN)** White blood cell whose nucleus is condensed into two to five segments connected by filaments. Distinguished from mononuclear cells such as monocytes and lymphocytes.
- selectin** Any of a family of cell adhesion molecules that mediate the binding of white blood cells and platelets to the vascular endothelium.
- senescent** Aging or growing old. A senescent red blood cell loses its deformability and is cleared by the spleen.
- sensitivity** In laboratory testing, the conditional probability that a person with a given disease will be correctly identified as having it by a clinical test (i.e., diagnostic sensitivity). Also, the lowest level of a substance that can be detected by a laboratory test procedure (i.e., analytical sensitivity).
- sepsis (septicemia)** Proliferation of pathologic organisms in the blood.
- sequestration** Transfer of blood cells from the circulation into a limited vascular area, such as the spleen. Platelets may be sequestered, which results in a decrease in their circulating numbers.
- serine protease** Any of a group of proteolytic enzymes of the trypsin family that include activated procoagulants (thrombin and factors VIIa, IXa, Xa, XIa, and XIIa) and activated inhibitors (antithrombin, activated protein C). Serine proteases are synthesized as inactive zymogens; activation occurs when the zymogen is cleaved at one or more specific sites by the action of another protease during the coagulation process.
- serine protease inhibitor (serpin)** Plasma proteins, for instance, antithrombin and heparin cofactor II, which control the coagulation cascade by inhibiting the serine proteases, particularly factors IIa and Xa.
- serotonin** Potent vasoconstrictor released from the dense granules of activated platelets.
- Sézary cell** Mononuclear cell with a cerebriform nucleus (resembling the surface of the cerebrum) and a narrow rim of cytoplasm. It is a characteristic finding in cutaneous T-cell lymphomas.
- Sézary syndrome** Cutaneous T-cell lymphoma characterized by exfoliative erythroderma, peripheral lymphadenopathy, and the presence of Sézary cells in the skin, lymph nodes, and peripheral blood.
- shift reticulocyte** Gray-blue red blood cell with increased diameter. The shift reticulocyte is a reticulocyte that has been released from the bone marrow prematurely to compensate for hemolytic anemia or acute blood loss. Shift reticulocytes require more than 1 day in the peripheral blood to lose residual RNA and gain a mature-looking reddish cytoplasm.
- sickle cell (drepanocyte)** Abnormal crescent-shaped red blood cell containing hemoglobin S, characteristic of sickle cell anemia.
- sickle cell anemia (sickle cell disease)** Severe chronic hemoglobinopathy in people who are homozygous for hemoglobin S. The abnormal hemoglobin results in distortion of red blood cells (sickle cells) and leads to crises characterized by joint pain, anemia, thrombosis, fever, and splenomegaly.
- sickle cell crisis** Any of several acute conditions occurring as part of sickle cell disease, such as aplastic crisis, which is temporary bone marrow aplasia; hemolytic crisis, which is acute red blood cell destruction; and vasoocclusive crisis, which is severe pain due to blockage of the blood vessels.
- sickle cell trait** Asymptomatic heterozygous condition characterized by the presence of both hemoglobin S and hemoglobin A.

- sideroblast** Bone marrow erythrocytic precursor that shows excessive iron granules (siderotic granules) with Prussian blue staining.
- siderocyte** Nonnucleated red blood cell in which particles of iron (siderotic granules) are visible with Prussian blue staining.
- siderotic granules (Pappenheimer bodies)** Red blood cell inclusions composed of ferric iron. With Prussian blue iron staining, they appear as multiple dark blue irregular granules. With Wright staining they appear as pale blue clusters.
- Southern blotting** Technique in which DNA fragments separated by gel electrophoresis are transferred to a nitrocellulose filter on which specific fragments can then be detected by their hybridization to probes.
- specificity** In laboratory testing, the conditional probability that a person who does not have a specific disease will be correctly identified as not having it by a clinical test (i.e., diagnostic specificity). Also used to describe the attribute of antibodies that are able to bind only with the antigen that stimulated their production.
- spectrin** Major cytoskeletal protein forming a lattice at the cytoplasmic surface of the cell membrane, providing lateral support to the membrane and thus maintaining its shape. Abnormalities in red blood cell spectrin account for hereditary spherocytosis, ovalocytosis, and pyropoikilocytosis.
- spherocyte** Abnormal spherical red blood cell with a decreased surface area-to-volume ratio. In Wright-stained peripheral blood films, spherocytes are dense, lack central pallor, and have a reduced diameter. Spherocytes appear most frequently in warm autoimmune hemolytic anemia and hereditary spherocytosis.
- spleen** Large organ in the upper left quadrant of the abdomen, just under the stomach. The spleen has the body's largest collection of macrophages, which are responsible for phagocytosis and elimination of senescent red blood cells. The spleen also houses many lymphoid cells.
- splenectomy** Excision of the spleen.
- splenomegaly** Enlargement of the spleen.
- standard deviation** Mathematic expression of the dispersion of a set of values or scores about the mean.
- standard precautions (universal precautions)** Practices to control blood-borne disease in which all human blood and body fluids are treated as if infectious. Infection is prevented by a series of protective methods including the use of sterile gloves, fluid-impermeable clothing, and eye protection.
- steatorrhea** Fat in the stool, usually due to malabsorption. Stool is green or colorless and foul smelling.
- stem cell** Undifferentiated mononuclear cell whose daughter cells may give rise to a variety of cell types and which is capable of renewing itself and thus maintaining a pool of cells that can differentiate into multiple other cell types.
- stomatocyte** Abnormal cup-shaped mature red blood cell that has a slitlike area of central pallor.
- storage pool deficiency** Inadequacy of platelet dense granules or dense granule contents that causes mucocutaneous bleeding. Usually hereditary and related to conditions with oculocutaneous albinism, such as Hermansky-Pudlak syndrome, Chédiak-Higashi syndrome, and Wiskott-Aldrich syndrome. Acquired storage pool disorder is sometimes associated with myelodysplastic syndrome.
- stroma** Supporting tissue or matrix of an organ.
- subcutaneous (SC)** Injected within the subdermal or dermal layer.
- sulfhemoglobin** Hemoglobin with a sulfur atom on one of its porphyrin rings, which makes it ineffective for transporting oxygen. Results from ingestion or exposure to drugs or chemicals containing sulfur.
- supernatant** Clear upper liquid part of a suspension after it has been centrifuged.
- suppuration** Formation or discharge of pus.
- supravital stain (vital stain)** Stain that colors living tissues or cells.
- surface-connected canalicular system (SCCS, open canalicular system, OCS)** System of channels that is distributed throughout platelets and extends the plasma membrane inward. The SCCS binds numerous coagulation factors and provides a route for secretion of the protein contents of α -granules.
- syncope** Brief lapse in consciousness; fainting.
- systemic bleeding disorder** Chronic episodic bleeding that is evidenced by bilateral petechiae and purpura, epistaxis, hematemesis, and menorrhagia. Indicates a primary coagulopathy such as thrombocytopenia, von Willebrand disease, or a qualitative platelet disorder.
- systemic lupus erythematosus (SLE)** Chronic autoimmune inflammatory disease manifested by severe vasculitis, renal involvement, and lesions of the skin and nervous system.
- T cell (T lymphocyte)** Lymphocyte that participates in cellular immunity, including cell-to-cell communication. The major T-cell categories are helper cells and suppressor-cytotoxic cells.
- target cell (codocyte)** Poorly hemoglobinized red blood cell (RBC) that is present in hemoglobinopathies, thalassemia, and liver disease. In a Wright-stained peripheral blood film, hemoglobin concentrates in the center of the RBC and around the periphery to resemble a "bull's-eye."
- teardrop cell (dacryocyte)** Red blood cell with a single pointed extension, resembling a teardrop. Dacryocytes are often seen in the myeloproliferative neoplasm called *myelofibrosis with myeloid metaplasia*.
- telangiectasia** Permanent dilation of capillaries, arterioles, and venules that creates focal red lesions, usually in the skin or mucous membranes.
- telomere** Repeating DNA sequences at a chromosome terminus.
- tetraploid** Possessing a double diploid chromosome complement, or four of each chromosome (4N).
- thalassemia** Production and hemolytic anemia characterized by microcytic, hypochromic red blood cells caused by deficient synthesis of α - or β -globin chains.
- thrombin** Primary serine protease of coagulation. Factors Xa and Va combine to cleave prothrombin to produce thrombin. Thrombin cleaves fibrinopeptides A and B from fibrinogen to initiate fibrin polymerization. Thrombin potentiates coagulation by activating platelets and factors XI, VIII, V, and XIII and also activates the coagulation control protein, protein C.
- thrombin clotting time (TCT, thrombin time, TT)** Coagulation test that measures the interval to clot formation after the addition of thrombin to plasma. Often used to test for the presence of heparin.
- thrombocyte** Platelet.
- thrombocytopenia** Abnormally high platelet count with dysfunctional platelets; seen in the myeloproliferative neoplasm known as *essential thrombocythemia*.
- thrombocytopenia** Platelet count below the lower limit of the reference interval, usually 150,000/ μ L.
- thrombocytosis** Platelet count above the upper limit of the reference interval, usually 450,000/ μ L.
- thrombophilia (hypercoagulability)** Abnormally increased tendency to clotting caused by a number of acquired or congenital factors.
- thrombopoietin** Hormone produced by renal tissue that recruits stem cells to the megakaryocyte cell maturation line and stimulates megakaryocyte mitosis and maturation in response to thrombocytopenia.
- thrombosis** Formation, development, or presence of a clot in a blood vessel (i.e., a thrombus).
- thrombospondin** Adhesive glycoprotein secreted by endothelial cells and platelet α -granules.
- thrombotic thrombocytopenic purpura (TTP)** Congenital or acquired deficiency of ADAMTS-13, an endothelial cell von Willebrand factor–cleaving protease. Ultra-large von Willebrand factor multimers activate platelets to form white clots in the microvasculature, causing severe thrombocytopenia with mucocutaneous bleeding, microangiopathic hemolytic anemia, and neuropathy.
- thromboxane A₂** Metabolically active product of the eicosanoid synthesis (cyclooxygenase, prostaglandin) pathway in platelets; binds platelet membrane receptor and activates platelets.
- thromboxane B₂** Metabolically inactive measurable plasma product of the eicosanoid synthesis (cyclooxygenase, prostaglandin) pathway.

- thrombus** In vivo blood clot causing vascular occlusion and tissue ischemia.
- tissue factor (TF)** Constitutive membrane protein of the subendothelium. Exposure to tissue factor activates factor VII and the tissue factor (extrinsic) coagulation pathway. Tissue factor is expressed on monocytes and endothelial cells in chronic inflammation.
- tissue plasminogen activator (TPA)** Serine protease that is secreted by endothelial cells and binds fibrin. It activates nearby plasminogen molecules to trigger fibrinolysis, with the formation of fibrin degradation products.
- toxic granulation** Presence of abnormally large, dark-staining, or dominant primary granules in neutrophils associated with bacterial infections.
- transcription** Process by which messenger RNA is produced from a DNA template.
- transferrin** Plasma iron-transport protein that moves iron from sites of absorption and storage to hematopoietic tissue for incorporation into developing normoblasts.
- transformed lymphocytes (atypical, variant, or reactive lymphocytes)** Lymphocytes whose altered morphology includes stormy blue cytoplasm and lobular or irregular nuclei. Variant lymphocytes indicate stimulation by a virus, particularly Epstein-Barr virus, which causes infectious mononucleosis.
- translation** Process by which the genetic information carried by nucleotides in messenger RNA directs the sequence of amino acids in the synthesis of a specific polypeptide.
- translocation** Rearrangement of DNA within a chromosome or transfer of a segment of one chromosome to a nonhomologous one.
- triploid** Possessing a single addition chromosome complement, resulting in three of each chromosome (3N).
- trisomy** Presence of an extra chromosome in addition to a homologous pair; for example, trisomy 21 in Down syndrome.
- tungsten-halogen light bulb** Bulb used in brightfield microscopy consisting of a tungsten filament enclosed in a small quartz bulb filled with halogen gas. Tungsten creates a very bright yellow light.
- unfractionated heparin (UFH, standard heparin)** Heparin extracted from porcine mucosa and purified to yield molecular weights ranging from 7000 to 15,000 Daltons. Used routinely in cardiac surgery. Unfractionated heparin therapy requires monitoring with the partial thromboplastin time or activated clotting time assay.
- universal precautions (now called standard precautions)** Practices to control blood-borne disease in which all human blood and body fluids are treated as if infectious. Infection is prevented by a series of protective methods including the use of sterile gloves, fluid-impermeable clothing, and eye protection.
- urobilinogen** Colorless water-soluble compound formed in the intestine through the breakdown of bilirubin by bacteria; low levels appear in the urine in healthy states.
- urokinase** Enzyme produced by the kidney endothelial cells that acts as a plasminogen activator.
- vacuole** Any clear space or cavity formed in the cytoplasm of a cell.
- vacuolization** Formation of vacuoles.
- vasoconstriction** Reduction in blood vessel diameter due to smooth muscle constriction.
- venipuncture** Use of a needle to puncture a vein and collect blood.
- vertigo** Sensation of rotation or movement of oneself or one's surroundings; dizziness.
- viscosity** Resistance of a liquid to flow.
- vital stain (supravital stain)** Stain that colors living tissues or cells.
- vitamin B₁₂ (cyanocobalamin)** Complex vitamin involved in the metabolism of protein, fats, and carbohydrate; normal blood formation; and nerve function.
- vitamin K** Natural phyloquinone occurring in green leafy vegetables and liver and produced by commensal intestinal organisms. Vitamin K catalyzes the γ -carboxylation of glutamic acid in a number of calcium-binding proteins, including the vitamin K-dependent coagulation proteins factors II (prothrombin), VII, IX, and X and control proteins C, S, and Z.
- vitamin K antagonist (VKA)** Substance that inhibits the action of vitamin K; for example, warfarin (Coumadin), which is used in oral anticoagulant therapy.
- von Willebrand disease** Congenital autosomal dominant variable mucocutaneous bleeding disorder characterized by a deficiency of von Willebrand factor activity and antigen, and subsequent impairment of platelet adhesion.
- waived testing** Test classification defined by the Clinical Laboratory Improvement Amendments that includes tests that are simple and accurate and can be performed by noncertified personnel.
- Waldenström macroglobulinemia** Form of monoclonal gammopathy in which IgM is overproduced by the clone of a plasma cell. Increased viscosity of the blood may result in circulatory impairment, and normal immunoglobulin synthesis is decreased, which increases susceptibility to infections.
- warfarin (Coumadin)** Vitamin K antagonist used as an anticoagulant to prevent thrombosis in people with atrial fibrillation, venous thromboembolism, or cardiac insufficiency. Also used prophylactically after orthopedic surgery. Warfarin suppresses vitamin K and reduces the activity of the vitamin K-dependent coagulation factors II (prothrombin), VII, IX, and X.
- warm antibody** IgG antibody that reacts optimally at a temperature of 37° C.
- warm autoimmune hemolytic anemia** Most common autoimmune hemolytic anemia, which results from the reaction of IgG autoantibodies with red blood cells at an optimal temperature of 37° C.
- Wiskott-Aldrich syndrome** Immunodeficiency disorder characterized by oculocutaneous albinism, thrombocytopenia, inadequate T- and B-cell function, and an increased susceptibility to viral, bacterial, and fungal infections.
- xanthochromic** Having a yellowish color. Used to describe cerebrospinal fluid, in which xanthochromia indicates the presence of bilirubin and thus serves as evidence of a prior episode of bleeding into the brain.
- X-linked** Pertaining to genes or to the characteristics or conditions they transmit that are carried on the X chromosome.
- X-linked recessive inheritance** Pattern of inheritance in which a recessive allele is carried on the X chromosome; results in the carrier state in females and development of disease characteristics in males, since they do not have a normal X chromosome to compensate.
- zymogen** Inactive precursor that is converted to an active form by an enzyme. Zymogens are inactive coagulation factors, such as prothrombin.

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Hematology/Hemostasis Reference Intervals (Continued from front cover)

Unless otherwise noted, data for reference interval tables were compiled from multiple sources and may vary slightly from intervals listed within chapters. Each laboratory must establish its particular intervals based on its instrumentation, methodology and demographics of the population it serves.

Body Fluid Cell Count Reference Intervals

Fluid	Reference Intervals
Cerebrospinal fluid	RBC: 0/ μ L WBC: 0–27/ μ L (0 to 30 d) 0–7/ μ L (31 d to 16 y) 0–5/ μ L (16 y to adult)
Synovial fluid	WBC: 0–200/ μ L, less than 25% neutrophils
Serous fluid	Dependent on source; usually less than 1000 total WBCs and less than 25% neutrophils

Coagulation Testing Reference Intervals and Therapeutic Target Ranges

COAGULATION REFERENCE INTERVALS (INFANTS TO AGE SIX MONTHS)					
Assay	Day 1	Day 5	Day 30	Day 90	Day 120
PT (seconds)	10.1–15.9	10.1–15.3	10.0–14.3	10.0–14.2	10.7–13.9
PTT (seconds)	31.3–54.3	25.4–59.8	32.0–55.2	29.0–50.1	26.6–40.3
Thrombin time (seconds)	19.0–28.3	18.0–29.2	19.4–29.2	20.5–29.7	19.7–30.3
Fibrinogen (mg/dL)	167–399	162–462	162–378	150–379	150–387
Factor VIII (%)	50–178	50–154	50–157	50–125	50–109
Factor IX (%)	15–91	15–91	21–81	21–113	36–136
VWF (%)	50–287	50–254	50–246	50–206	50–197
Antithrombin (%)	39–87	41–93	40–108	73–121	84–124
Protein C (%)	17–53	20–64	21–65	28–80	37–81
Protein S (%)	12–60	22–78	33–93	54–118	55–119

Andrew M, et al. Development of the hemostatic system in the neonate and young infant. *Am J Pediatr Hematol Oncol* 1990;12:95–104.

COAGULATION REFERENCE INTERVALS (PEDIATRIC POPULATION AGES 7–17)					
Assay	7–9	10–11	12–13	14–15	16–17
PT (seconds)	13.0–15.4	13.0–15.6	13.0–15.2	12.8–14.5	12.6–15.7
PTT (seconds)	27–38	27–38	27–39	26–36	26–35
Factor VIII (%)	76–199	80–209	72–198	69–237	63–221
Factor IX (%)	70–133	72–149	73–152	80–161	86–176
VWF activity (%)	52–176	60–195	50–184	50–203	49–204
VWF antigen (%)	62–180	63–189	60–189	57–199	50–205

Flanders MM, et al. Pediatric reference intervals for seven common coagulation assays. *Clin Chem* 2005;51:1738–42.

COAGULATION REFERENCE INTERVALS (ADULTS)	
Clot-based Coagulopathy Screening Tests	
PT	12.6–14.6 seconds
PTT	25–35 seconds
Thrombin time	≤ 21 seconds
Coagulation Factor Assays	
Fibrinogen	220–498 mg/dL
Coagulation factors II, V, VII, IX, X, XI, XII	50–150%
Coagulation factor VIII	50–186%
Coagulation factor XIII; immunoassay	59–192%
Von Willebrand factor activity; ristocetin cofactor	50–166%
Von Willebrand factor activity; collagen binding assay	50–166%
Von Willebrand factor antigen; immunoassay	50–249%
HMW kininogen (Fitzgerald factor) and prekallikrein (Fletcher factor)	65–135%
Coagulation Control Proteins; Thrombosis Risk Assays	
Antithrombin activity	78–126%
Protein C activity	70–140%
Protein S activity	65–140%
Activated protein C resistance	ratio >1.8
Anti-cardiolipin antibody, IgG	<12 IgG phospholipid units (GPL)
Anti-cardiolipin antibody, IgM	<10 IgM phospholipid units (MPL)
Anti-β2 glycoprotein 1, IgG and IgM	< 20 anti-β2 glycoprotein 1 units (GU)
PTT-based lupus anticoagulant phospholipid correction study	Shortened by >8 seconds
Dilute Russell viper venom phospholipid correction study	Shortened by a ratio >1.3
D-dimer, quantitative immunoassay	110–240 ng/mL
Plasminogen activator inhibitor-1	14–28 mg/dL
Homocysteine	Males 4.3–9.9; females 3.3–7.2 mmol/L

Adult coagulation reference intervals are from the University of Alabama at Birmingham Hospital special coagulation laboratory and are certain to vary by institution.

THERAPEUTIC TARGET RANGES (ADULTS)	
Monitoring Warfarin (Coumadin) Therapy	
Prothrombin time INR for most indications	2.0–3.0
Prothrombin time INR for treating mechanical heart valves and lupus anticoagulant with recurrent thrombosis	2.5–3.5
Warfarin; chromogenic factor X (ten) assay	22–40%
Monitoring Heparin Therapy	
Prophylactic LMW heparin; anti-FXa heparin assay	0.2–0.4 heparin units/mL
Therapeutic unfractionated heparin, anti-FXa heparin assay	0.3–0.7 heparin units/mL
Therapeutic LMW heparin, twice daily; anti-FXa heparin assay	0.5–1.0 heparin units/mL
Therapeutic LMW heparin, once daily; anti-FXa heparin assay	1.0–2.0 heparin units/mL
Prophylactic fondaparinux pentasaccharide; anti-FXa heparin assay	0.2–0.4 mg/mL
Therapeutic fondaparinux pentasaccharide; anti-FXa heparin assay	0.5–1.5 mg/mL
Therapeutic unfractionated heparin; PTT	Consult laboratory director for current range, never use a generally published target range

Target therapeutic ranges are based on international practice standards.