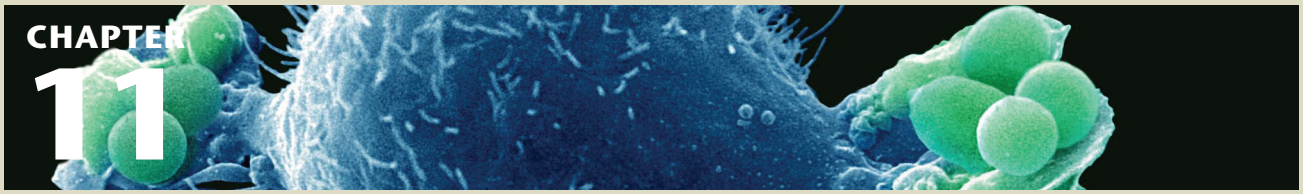

CHAPTER 11

Laboratory hematology

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General concepts

Hematology laboratory tests are ordered and interpreted within the context of a specific patient, for example, a routine screening or preoperative assessment, or in the setting of an illness for diagnosis or follow-up. Clinical judgment is applied in both the selection of tests and in their interpretation. Some unexpected results may require confirmation, particularly if there is a question about the integrity of the specimen (eg, heparin contamination, wrong collection tube or volume of blood, delayed processing). Additional causes of inaccurate laboratory results include sample mislabeling, analytical mistakes, and reporting errors.

Terminology

Sensitivity, specificity, and positive or negative predictive values are defined using the following clinical variables: true positive (TP; assay correctly identifies a condition in those who have it), false positive (FP; assay incorrectly identifies disease when none is present), true negative (TN; assay correctly excludes a disease in those without it), and false negative (FN; assay incorrectly excludes disease when it is present).

Sensitivity [TP/(TP + FN) × 100] is the ability of a test to detect a true abnormality; as the sensitivity of a test increases, the risk of an FP result increases (increasing sensitivity comes at the cost of decreasing specificity). Very sensitive tests are helpful for screening, by ruling out a diagnosis or disease when the test is negative (high negative predictive value).

Specificity [TN/(TN + FP) × 100] is the ability of a test to detect a normal result if the abnormality is not present; as the specificity increases, the risk of an FN result increases. Specific tests are useful for confirmation, by ruling in a diagnosis or disease when the test is positive (high positive predictive value).

Precision is reproducibility of a value during repeated testing of a sample.

Accuracy is the ability of a test to obtain the assigned value of an external standard (run as though it were a clinical sample).

Predictive value is the likelihood that an abnormal test indicates a patient with the clinical abnormality (*positive predictive value;* [TP/(TP + FP) × 100]) or the likelihood that a normal test indicates a patient without the abnormality (*negative predictive value;* [TN/(TN + FN) × 100]). Positive and negative predictive values depend on the frequency of the abnormality being sought in the population as well as the sensitivity and specific of the laboratory methods.

Reference ranges are derived from a sample of a well population and typically reflect the results of 95% (mean ±2 standard deviation [SD]) of disease-free individuals. The reference ranges of some assays are determined by the results of 98%–99% of disease-free individuals (mean ±3–5 SD).

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Specific laboratory tests

Automated blood cell counting

In addition to complete blood counts (CBCs) and five-part leukocyte differential counts (LDCs), hematology analyzers

provide quantitative and qualitative information about reticulocytes, nucleated red blood cells (RBCs), and platelets. Additional information, such as platelet immaturity, extended leukocyte counts, and reticulocyte-specific indices, is available only from selected instrument manufacturers. Because of the large number of cells counted from each blood sample and analysis using multiple physical principles and sophisticated software, hematology analyzers generally produce accurate and precise CBCs and LDCs, with the exception of basophils, because of their low frequency. Many laboratories no longer report band neutrophils, because accurate and precise identification by automated and morphologic techniques is poor, and their clinical significance, if any, appears minimal with the possible exception of neonatal sepsis. Hematology analyzers provide excellent sensitivity to distinguish between normal and abnormal samples via operator alerts prompting microscopic review of a stained peripheral blood smear for selected samples. As a result, approximately 30% of hospital patients' samples require review of a stained blood smear.

Automated blood cell counters use various technologies to enumerate and classify blood cells (Figure 11-1). Most platforms available for clinical use utilize at least two of the following techniques.

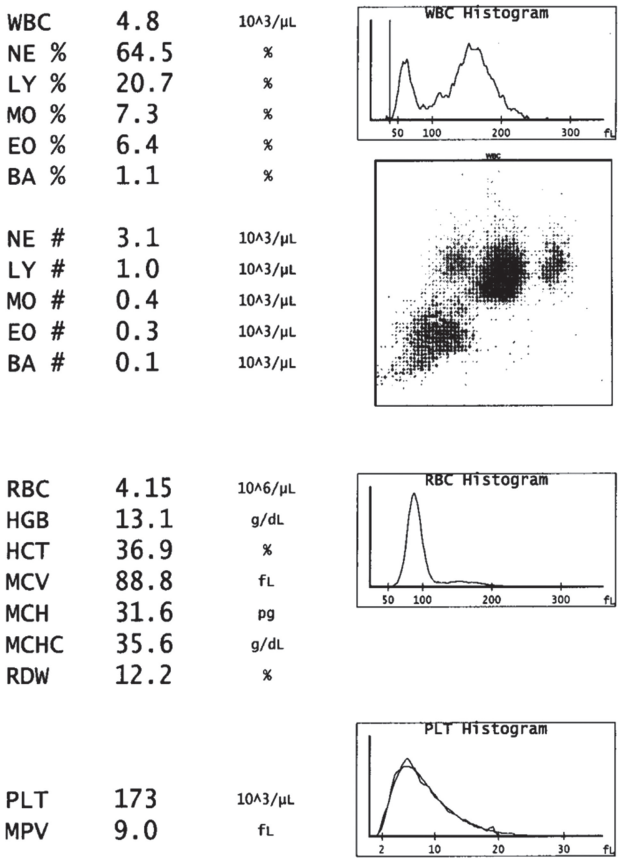

Figure 11-1 Data and histograms performed on a Beckman-Coulter LH 750 automated hematology analyzer from a healthy adult. The white blood cell (WBC), red blood cell (RBC), and platelet (PLT) histograms represent cell volumes determined by impedance. The second histogram from the top displays WBC light scatter in a flow cell; the y-axis indicates forward scatter and volume, and the x-axis indicates side scatter due to granularity and nuclear features. Basophils (BA) are detected by an alternative physical property not displayed. EO = eosinophil; HCT = hematocrit; HGB = hemoglobin; LY = lymphocyte; MCH = mean corpuscular hemoglobin; MCHC = mean corpuscular hemoglobin concentration; MCV = mean corpuscular volume; MO = monocyte; MPV = mean platelet volume; NE = neutrophil; RDW = red blood cell distribution width.

Aperture impedance (Coulter Principle)

Cells diluted in a conducting solution are counted, and their volume is determined by measuring change in electrical resistance as they flow through a narrow aperture and interrupt a direct electrical current. Software analysis defines RBCs, white blood cells (WBCs), and platelets based on volume limits and calculates RBC and platelet indices.

Optical absorbance

This technique exploits the cytochemical reaction of an intracellular enzyme, such as myeloperoxidase, to absorb white light from a tungsten light source after the addition of a substrate. Light absorbance is proportionate to the intensity of the enzyme-catalyzed reaction. This technique may be used to detect and distinguish peroxidase-containing cell types (neutrophils, eosinophils, monocytes) from peroxidase-negative lymphocytes and basophils.

Optical light scatter

This method monitors the light-scattering properties of blood cells, using a technique similar to that employed by flow cytometers. Cells pass in single file across the path of a unifocal laser. The amount of light scattered at a low angle from the incident light path is proportional to cell volume. The amount of light scattered at a wide angle depends on such factors as cytoplasmic granules and nuclear shape. All of the major hematology analyzer manufacturers use light-scattering technology.

Fluorescence

In addition to the physical properties of cells, fluorochrome-labeled antibodies recognizing cell surface or intracellular epitopes further refine the separation of individual cell types. A variety of reagents can be used to distinguish platelets (thiazole orange, anti-CD41, anti-CD42b, anti-CD61), reticulocytes (thiazole orange, anti-CD4K, RNA dyes), fetal RBCs

(antihemoglobin F/D), nucleated RBCs (propidium iodide, Draq 5, other DNA-binding dyes), neutrophils, lymphocytes, and blasts.

Erythrocyte analysis

Automated blood cell counters measure the number (RBC count, reported in units of 10^6/mL) and volume (mean corpuscular volume [MCV], reported in units of 10^{-15} L) of RBCs, and hemoglobin concentration (reported in units of g/dL) after lysing red blood cells; all other parameters are calculated. Hemoglobin is converted by potassium ferricyanide to cyanmethemoglobin, and absorbance is measured by a spectrophotometer at 540 nm. Some analyzers use a cyanide-free method. RBCs may be spuriously increased in patients with hyperleukocytosis and giant platelets, and decreased in the presence of RBC agglutinins, cryoglobulins, and in vitro hemolysis. Hemoglobin measurement can be elevated artifactually by increased sample turbidity because of leukocytosis, paraproteinemia, carboxyhemoglobinemia, hyperbilirubinemia, or hyperlipidemia.

MCV is calculated from the distribution of individual RBC volumes. This measurement can be elevated artifactually by agglutination of RBCs, resulting in measurement of more than one cell at a time; hyperglycemia, causing osmotic swelling of the RBC; and spherocytes, which have decreased deformity.

Automated hematocrit (%) is calculated by multiplying the MCV by the RBC number: hematocrit = MCV (10^{-15} L) × red blood cells ($3\ 10^{12}$/L) × 100.

The mean corpuscular hemoglobin (MCH) is expressed in picograms (10^{-12} g). The MCH is calculated by dividing hemoglobin (g/L) by red blood cell count (10^{12}/L). An elevated MCH can be an artifact of increased plasma turbidity.

The MCH concentration (MCHC) is expressed in grams of hemoglobin per deciliter of packed RBCs. The MCHC is calculated by dividing the hemoglobin concentration (g/dL) by the hematocrit (%) × 100. Any artifact affecting the hematocrit or hemoglobin determinations can alter the MCHC.

The red blood cell distribution width (RDW) is the coefficient of variation of RBC size (anisocytosis): standard deviation/MCV. The RDW is used in the evaluation of anemia. The RDW is more frequently elevated with microcytic anemias due to iron deficiency anemia than to thalassemia or anemia of chronic disease, and also is elevated more frequently with macrocytic anemias due to vitamin B12 or folate deficiency than to liver disease, hypothyroidism, or a reticulocytosis. Myelodysplastic syndromes, such as refractory anemia, or RBC transfusions to patients with severe microcytic or macrocytic anemias can produce a dimorphic RBC pattern with a very wide RDW.

Reticulocyte counts

Automated hematology analyzers use dyes to detect residual mRNA in young erythrocytes, and all provide accurate reticulocyte counts expressed as a percentage of RBCs or as an absolute number. Some blood cell counters provide reticulocyte indices that are analogous to the standard RBC indices, including reticulocyte hemoglobin content (CHr) on Advia analyzers (Siemens, Tarrytown, NY) and reticulocyte MCV (MCVr) on several others. Reductions in CHr and MCVr reflect inadequate hemoglobin synthesis in real time, providing immediate information about functional iron deficiency when other biochemical markers of iron availability may be difficult to interpret due to inflammatory conditions. CHr is particularly useful for assessing response to erythropoiesis-stimulating agents and iron therapy in renal dialysis patients.

Nucleated red blood cells

Circulating nucleated red blood cells (NRBCs) occur in newborns; however, beyond this period, the presence of NRBCs is abnormal and is associated with various hematopoietic stresses, including hemolytic anemias, myeloproliferative disorders, metastatic cancer to bone marrow, and hypoxia. All major hematology analyzer brands enumerate NRBCs and correct WBC and lymphocyte counts for interference from NRBCs analyzer.

Leukocyte analysis

To differentiate lymphocytes, monocytes, neutrophils, eosinophils, and basophils, most instruments use impedance and or light scattering, plus additional physical properties. Coulter and Sysmex use radiofrequency conductivity, and Advia (Siemens) uses peroxidase staining. Differentials typically are reported as percentages of WBC and as absolute counts. Automated blood cell counters provide sensitive flags and warnings for immature granulocytes and monocytes and abnormal lymphocytes. Instrument manufacturers continue to refine technologies to report extended differentials to quantify neutrophil precursors, including metamyelocytes, myelocytes, promyelocytes, and blasts. Some Sysmex analyzers identify a subset of WBCs called hematopoietic progenitor cells, which correlate with CD34 counts and can be used to monitor peripheral stem cell mobilization.

Platelet analysis

Automated blood cell counters enumerate platelets, measure volume, and calculate mean platelet volume (MPV). Associations between MPV and acquired mechanisms of thrombocytopenia suggest that MPV increases with peripheral

destruction of platelets because of increased megakaryocyte ploidy and production of larger platelets, whereas MPV decreases when platelet production is suppressed. Platelets undergo time-dependent shape changes when exposed to ethylenediaminetetraacetic acid (EDTA), however, leading to inaccurate MPV results and thus diminishing its clinical utility. Inaccurate automated platelet counts can result from fragmented RBCs, congenital (inherited macrothrombocytopenia disorders such as May-Hegglin anomaly) or acquired (myeloproliferative disorders or idiopathic thrombocytopenic purpura) macrothrombocytes, and EDTA-mediated platelet clumping because of immunoglobulin M (IgM) autoantibodies. Hematology analyzers provide sensitive warnings for abnormal platelet populations requiring manual smear review to confirm or revise platelet counts. Analogous to reticulocytes, young platelets contain detectable mRNA. Currently, only certain analyzers provide an immature platelet fraction (IPF), with a reference range of 1.1%-6.1%, based on the analysis of cell volume and fluorescence intensity of mRNA binding dye. Potential applications include differentiating thrombocytopenia due to megakaryopoiesis failure from peripheral destruction and determining earlier evidence of marrow regeneration following stem cell transplantation or response to a thrombopoietin mimetic drug.

Examination of peripheral blood smears

Blood smears are stained with either the Wright or the May-Grunwald-Giemsa stains and can be prepared by automated slide maker or strainers, which can be interfaced with hematology analyzers. Microscopic examination of stained blood smears can identify morphologic abnormalities that automated hematology analyzers nonspecifically flag or, rarely, miss. The examination begins at low power (×10), scanning for platelet clumps or abnormal, large, nucleated cells that may be located along the lateral and leading edges of the smear. At higher magnification (×50 and ×100), the optimal area to examine RBC, platelet, and leukocyte morphologies and to perform WBC differentials is the transitional area between the thick part of the smear and the leading edge (Table 11-1), where there are only a few overlapping RBCs and central pallor of normal red blood cells is evident. Hematologists should review a patient's peripheral smear as part of any consultation potentially involving qualitative or quantitative blood cell abnormalities.

The accuracy of manual WBC differentials suffers from small sample size (typically 100 cells), distributional bias of WBCs on the smear, and variable interobserver agreement regarding cell classification. Advances in digital microscopy and image analysis can improve the accuracy of WBC classification while reducing technical time. For example, the CellaVision DM96 (CellaVision, Lund, Sweden) scans a stained blood smear, makes digital images of WBCs, classifies them, and presents the sorted WBC images to an operator to confirm or reclassify. When compared with manual differentials, automated morphologic differentials demonstrate excellent routine differential accuracy and sensitivity to detect blasts. In addition, stored images can be reviewed at remote locations, such as outpatient clinics.

Supravital stains are used to detect RBC inclusions. Crystal violet detects denatured hemoglobin inclusions (Heinz bodies) because of enzymopathies such as glucose-6-phosphate dehydrogenase (G6PD) deficiency; brilliant cresyl blue is used to precipitate and detect unstable hemoglobins (hemoglobin H cells in α-thalassemias).

Bone marrow aspirate and biopsy

Following are the most frequent indications for bone marrow biopsy: unexplained cytopenias; unexplained leukocytosis, erythrocytosis, or thrombocytosis; staging of lymphoma and some solid tumors (particularly in patients with cytopenias or other findings suggestive of bone marrow involvement); diagnosis of plasma cell neoplasms (myeloma and monoclonal gammopathy of uncertain significance); evaluation of systemic iron levels; and unexplained splenomegaly. Bone marrow aspirate and biopsy commonly are performed by collecting specimens from the posterior iliac crests. Bone marrow aspirates also can be obtained from the sternum. In newborns and young infants, marrow aspirates often are obtained from the anterior tibia. Quality smears require adequate spicule harvesting because perispicular areas are the most representative areas to examine.

The bone marrow aspirate and touch prep are stained with either the Wright or May-Grunwald-Giemsa stains; unstained smears should be retained and kept frozen for possible special stains. The aspirate is used for cytologic examination of the bone marrow cells and for performing the differential. Bone marrow core biopsies are fixed in formalin, and the biopsy specimen is decalcified and embedded in paraffin; 3-4 mm sections are then cut and stained with hematoxylin and eosin or Giemsa stains.

When examining pediatric marrows, it is understood that erythroid hyperplasia is present at birth because of high levels of erythropoietin. Lymphocytes may compose 40% of the marrow cellularity in children <4 years of age, and eosinophils are present in higher numbers than in adults.

Perls or Prussian blue reactions are used to identify hemosiderin in nucleated red blood cells (sideroblastic iron) and histiocytes (reticuloendothelial iron). See Table 11-2 for other cytochemical stains.

Ringed sideroblasts are abnormal nucleated red cells with blue-staining iron granules surrounding at least two-thirds of the nucleus. These iron granules are present in mitochondria

Table 11-1 Red blood cell abnormalities.*

Abnormality	Description	Cause	Disease association
Acanthocytes (spur cells)	Irregularly spiculated red cell	Altered membrane lipids	Liver disease, abetalipoproteinemia, postsplenectomy
Basophilic stippling	Punctate basophilic inclusions	Precipitated ribosomes	Lead toxicity, thalassemias
Bite cells (degmacyte)	Smooth semicircle taken from one edge	Heinz body pitting by spleen	G6PD deficiency, drug-induced oxidant hemolysis
Burr cells (echinocytes)	Short, evenly spaced spicules	May be related to abnormal membrane lipids	Usually artifactual, uremia, bleeding ulcers, gastric carcinoma
Cabot ring	Circular, blue, threadlike inclusion with dots	Nuclear remnant	Postsplenectomy, hemolytic anemia, megaloblastic anemia
Howell-Jolly bodies	Small, discrete basophilic dense inclusions; usually single	Nuclear remnant	Postsplenectomy, hemolytic anemia, megaloblastic anemia
Pappenheimer bodies	Small dense basophilic granules	Iron-containing siderosomes or mitochondrial remnant	Sideroblastic anemia, postsplenectomy
Schistocytes (helmet cells)	Distorted, fragmented cell, with 2-3 pointed ends	Mechanical distortion in the microvasculature by fibrin strands; damage by mechanical heart valves	Microangiopathic hemolytic anemia, prosthetic heart valves, severe burns
Spherocytes	Spherical cell with dense appearance and absent central pallor; usually decreased diameter	Decreased membrane redundancy	Hereditary spherocytosis, immunohemolytic anemia
Stomatocytes	Mouth- or cuplike deformity	Membrane defect with abnormal cation permeability	Hereditary stomatocytosis, immunohemolytic anemia
Target cell (codocyte)	Targetlike appearance, often hypochromic	Increased redundancy of cell membrane	Liver disease, postsplenectomy, thalassemia, HbC
Teardrop cell (dacryocyte)	Distorted, drop-shaped cell		Myelofibrosis, myelophthisic anemia

*Blood smear abnormalities can be artifacts of poor slide preparation or viewing the wrong part of the smear.
G6PD = glucose-6-phosphate dehydrogenase; HbC = hemoglobin C.
Modified from Kjedsberg C, ed. *Practical Diagnosis of Hematologic Disorders*. 2nd ed. Chicago, IL: ASCP Press; 1995.

Table 11-2 Cytochemical stains.

Cytochemical stain	Substrate and staining cells
Myeloperoxidase	Primary granules of neutrophils and secondary granules of eosinophils. Monocytic lysosomal granules stain faintly.
Sudan black B	Stains intracellular phospholipids and other lipids. Pattern of staining is similar to myeloperoxidase.
Naphthol AS-D chloroacetate esterase (specific esterase)	Granulocytes stain; monocytes do not stain. Can be used in biopsies to stain granulocytes and mast cells.
α-Naphthyl butyrate (nonspecific esterase)	Stains monocytes, macrophages, and histiocytes. Does not stain neutrophils.
α-Naphthyl acetate (nonspecific esterase)	Megakaryocytes stain with α-naphthyl acetate but not α-naphthyl butyrate.
Terminal deoxynucleotidyl transferase (TDT)	Intranuclear enzyme. Stains thymocytes and lymphoblasts. Some myeloblasts stain positively.
Tartrate-resistant acid phosphatase (TRAP)	Stains an acid phosphatase isoenzyme. Positive staining in hairy cell leukemia, Gaucher cells, activated T-lymphocytes.
Periodic acid-Schiff (PAS)	Detects intracellular glycogen and neutral mucosubstances. Positive staining in acute lymphoblastic leukemia, acute myeloid leukemia, erythroleukemia, and Gaucher cells.
Toluidine blue	Detects acid mucopolysaccharides. Positive in mast cells and basophils.
Tryptase	Positive in mast cells, negative in basophils. Mast cells in systemic mast cell disease frequently have a spindled shape.

surrounding the nuclear membrane. Iron staining of the biopsy can underestimate the marrow iron stores because of the loss of iron during decalcification.

Immunohistochemical stains

A large array of specific antibodies detected by enzymatic formation of a colored product linked to the antigen–antibody complex are now available for use on blood smears, marrow aspirates, and bone marrow biopsies or other tissues. Many cytochemical stains, such as tartrate-resistant acid phosphatase (TRAP) and myeloperoxidase, have been converted into immunohistochemical (IHC) reactions.

IHC stains are used on marrow aspirates and blood smears as an alternative or adjunct to flow cytometry. The advantage of immunohistochemistry is the ability to correlate morphology with phenotype. IHC can be used to phenotype undifferentiated tumors, lymphoproliferative disorders, and atypical lymphoid infiltrates. In patients whose marrow cannot be aspirated (dry tap), immunohistochemistry can be performed on the biopsy section. IHC also can be used on sections of lymph nodes or other tissues when there is concern about lymphoma or some other neoplastic disease.

Preparation of bone marrow samples for ancillary studies

Bone marrow collected in EDTA is adequate for both flow cytometry and molecular analysis. Bone marrow collected for cytogenetic studies should be collected in a sterile tube containing tissue culture medium such as RPMI (containing fetal bovine serum, L-glutamine, and antibiotics) and anticoagulant.

Paraffin-embedded tissue can be used for polymerase chain reaction (PCR) of genomic DNA sequences. Reverse transcriptase PCR (RT-PCR) assays require that RNA preparations be performed as early as possible to prevent digestion by ubiquitous nucleases.

Flow cytometry

The most common applications of flow cytometry in hematology include the detection of cell surface or cytoplasmic proteins using fluorescent-labeled monoclonal antibodies or the assessment of DNA content using DNA-binding dyes.

Flow cytometry is used for phenotyping populations of cells, enumerating early progenitors for stem cell transplantations, detecting minimal residual disease, detecting targets for immunotherapy, and assessing the presence of prognostic markers. See Table 11-3 for a summary of clinical uses of flow cytometry in ancillary studies.

Gating is necessary to identify cells of interest in a mixed population of cells. Three major leukocyte populations (lymphocytes, monocytes, and neutrophils) can be defined using light scatter. Forward-angle scatter (FS; low angle) measures cell size, and side-light scatter (SS) measures internal cellular granularity. Lymphocytes have the lowest FS and SS signals, monocytes have intermediate FS and SS signals,

Table 11-3 Specimen allocation for ancillary studies.

Clinical problem	Ancillary techniques
Pancytopenia	Flow cytometry (LGL, hairy cell leukemia, PNH clone, AML) Cytogenetics (AML, MDS)
	Molecular genetics
Myeloid leukemia	Flow cytometry (phenotyping)
	Cytogenetics and FISH
	Molecular genetics (*BCR-ABL, PML/RARA, AML1/ETO*)
Lymphoproliferative disorder	Flow cytometry (phenotyping, prognostic markers such as ZAP-70)
	Cytogenetics: t(1;19) in pre–B-cell ALL, t(14;18) in follicular lymphomas, etc.
	FISH
	Molecular genetics (clonality, specific markers such as *BCL2, BCL6,* etc.)
	Immunohistochemistry (phenotyping, prognostic markers)
Myeloproliferative disorders	Cytogenetics
	FISH (*BCR-ABL*)
	Molecular genetics (*BCR-ABL, JAK2*)
Plasmaproliferative disorders	Flow cytometry (phenotyping, labeling index)
	Cytogenetics

ALL = acute lymphoblastic leukemia; AML = acute myelogenous leukemia; CLL = chronic lymphocytic leukemia; FISH = fluorescence in situ hybridization; LGL = large granular lymphocyte leukemia; MDS = myelodysplastic syndrome; PNH = paroxysmal nocturnal hemoglobinuria.

and neutrophils have high SS and slightly lower FS signals. Blasts generally have low FS and SS.

The most common method for gating different cell populations is by plotting right-angle SS against CD45. Cells can be separated based on the intensity of staining they display with the conjugated antibody that is classified as either bright or dim. Lymphocytes are bright CD45 and have a low SS signal, neutrophils are dim to moderately bright CD45 and have a high SS signal, and monocytes are bright CD45 and have an intermediate SS. Blasts have low SS and dim to negative CD45 expression, the latter being more common in blasts of lymphoid lineage.

Flow cytometry also can be used to detect populations of natural killer (NK) cells. NK cells express CD2, CD7, CD16, and CD56 and show variable expression of CD57 and CD8. NK cells do not express CD3, and the absence of CD3 expression can be used to differentiate NK cells from T-cells.

In addition to determining cell lineage, flow cytometry can be used to detect prognostic markers. For example, flow cytometric analysis of the tyrosine kinase ZAP-70 can be used to subdivide chronic lymphocytic leukemia (CLL) into prognostic groups. Positivity for ZAP-70 is highly correlated with unmutated DNA, a feature of CLL arising from pre–germinal center cells, and patients with pre–germinal center CLL have a decreased overall survival when compared with patients with CLL arising from post–germinal center cells. Positivity for CD38 by flow cytometric analysis also is correlated with unmutated DNA, but the correlation is not as strong as it is with ZAP-70.

Uncommitted hematopoietic progenitors are $CD34^+$ and $CD38^-$; expression of CD38 is evidence of lineage commitment. Myeloid maturation is characterized by the following maturational sequence: colony forming units–erythroid granulocyte, macrophage, and megakaryocyte (CFU-GEMM, CD34+, MHC class II+, CD33 –/+); and followed by colony forming unit–granulocyte, macrophage (CFU-GM, CD34+, MHC class II+, CD33+, CD13 –/+, CD15 –/+). Neutrophil precursors then progressively lose MHC class II and CD33 and gain CD11b, CD16, and CD32. Monocytes retain expression of MHC class II and CD33 and also gain expression of CD14 and CD64.

Appearance of CD71, loss of CD34 and CD33, and decreased expression of CD45 characterize erythroid maturation. With further differentiation, CD71 expression decreases, glycophorin expression increases, and CD45 disappears.

Megakaryocytic differentiation is indicated by the expression of glycoprotein (GP) IIb (CD41). GPIIb-IIIa (CD61) expression increases as CD34 expression decreases. GPIb (CD42b) is expressed at the promegakaryocyte stage. GPV (CD42d) expression increases with megakaryocyte differentiation.

B- and T-cell precursors express terminal deoxynucleotidyl transferase (TDT), human leukocyte antigen (HLA)-DR, and CD34. B-cell differentiation is indicated by the expression of CD19 followed by CD10. As B-cells mature, they lose cell surface expression of CD34 and CD10 and express IgM on the cell surface. Expression of surface IgM is associated with the expression of mature B-lymphocyte markers (CD20, CD21, CD22, and CD79b). Mature B-cells express an immunoglobulin heavy chain, such as IgM, and either the κ- or a λ-light chain. A predominant expression of one type of light chain on the cell surface of a population of B-cells is known as light-chain restriction and is indicative of a monoclonal process.

T-cell precursors express TDT, HLA-DR, and CD34. Differentiation is indicated by the expression of cytoplasmic CD3 and CD7, followed by the expression of CD2 and CD5. The common thymocyte also expresses CD1, CD4, and CD8. The mature helper or inducer lymphocyte expresses CD2, CD3, CD4, and CD5 and may express CD7. The mature suppressor or cytotoxic T-lymphocyte expresses CD2, CD3, CD4, CD5, and CD8 and may express CD7. T-cell neoplasms may be associated with abnormal expression patterns of T-cell antigens, and the abnormal pattern may be detected by flow cytometric analysis. See Tables 11-4 through 11-10 for useful CD markers.

Flow cytometry can be used to diagnose paroxysmal nocturnal hemoglobinuria (PNH). PNH is associated with the absence of glycosylphosphatidylinositol (GPI)-anchored membrane proteins, including two complement regulatory molecules: decay accelerating factor (DAF, CD55) and protectin (MIRL, CD59). The absence of these proteins from the cell surface of erythrocytes can be detected by flow cytometry using antibodies to CD55 and CD59, respectively. Alternatively, PNH granulocytes are detected by the absence of GPI anchor binding by FLAER, an Alexa® 488 labeled variant of aerolysin. Flow cytometry technology can discriminate between fetal and adult red cells or Rh^+ and Rh^- red cells during pregnancy and postpartum and can identify red cell skeletal disorders, such as hereditary spherocytosis.

Cytogenetics

Cytogenetic analysis can be performed from cultured (indirect) and uncultured (direct) preparations. In the indirect assay, cells are grown so that mitotic forms can be visualized and distinct chromosomal banding patterns can be assessed (conventional cytogenetics). Growing or culturing the cells increases the mitotic rate and improves chromosome morphology. Mitogens may be useful in improving the yield of karyotyping abnormal cells and are particularly useful when analyzing mature B- or T-cell processes.

Table 11-4 Clinically useful CD markers.

Marker	Lineage association
Progenitor cells	
CD34	Progenitor cells, endothelium
CD38	Myeloid progenitors, T, B, NK cells, plasma cells, monocytes, CLL subset
B-cell markers	
CD10	Pre-B-lymphocytes, germinal center cells, neutrophils
CD19	B-cells (not plasma cells or follicular dendritic cells)
CD20	B-cells (not plasma cells)
CD21	Mature B-cells, follicular dendritic cells, subset of thymocytes
CD22	Mature B-cells, mantle zone cells (not germinal center cells)
CD23	B-cells, CLL
CD79b	B-cells (not typical CLL)
CD 103	Intraepithelial lymphocytes, hairy cell leukemia, T-cells in enteropathic T-cell lymphoma
FMC7	B-cells (not typical CLL), hairy cell leukemia
T-cell markers	
CD2	Pro- and pre-T-cells, T-cells, thymocytes, NK cells, some lymphocytes in CLL and B-ALL
CD3	Thymocytes, mature T-cells, cytoplasm of immature T-cells
CD5	Thymocytes, T-cells, B-cells in CLL, B-cells in mantle cell lymphoma
CD4	Helper T-cells, monocytes, dendritic cells, activated eosinophils, thymocytes
CD7	Pro- and pre-T-cells, T-cells, thymocytes, NK cells, some myeloblasts
CD8	Suppressor T-cells, NK cells, thymocytes
CD25	Activated T- and B-cells, adult T-cell leukemia/lymphoma
NK/cytotoxic T-cell markers	
CD16	NK cells, monocytes, macrophages, neutrophils
CD56	NK cells, myeloma cells
CD57	NK cells, T-cell subset
Myeloid and monocytic markers	
CD13	Monocytes, neutrophils, eosinophils, and basophils
CD14	Monocytes, macrophages, subset of granulocytes
CD33	Myeloid lineage cells and monocytes
CD117	Immature myeloid cells, AML
Monocytes	
CD11c	Monocytes, macrophages, granulocytes, activated B- and T-cells, NK cells, hairy cell leukemia
CD15	Myeloid lineage cells and monocytes
CD64	Monocytes, immature myeloid cells, activated neutrophils
Megakaryocytic markers	
CD41	Platelets and megakaryocytes (GPIIb)
CD42	Platelets and megakaryocytes (CD42a: GPI; CD42b: GPIb)
CD61	Platelets, megakaryocytes, endothelial cells (GPIIb-IIIa)
Erythroid markers	
CD71	Transferrin receptor is upregulated upon cell activation
CD235a	Glycophorin A

AML = acute myelogenous leukemia; B-ALL = B-lineage acute lymphoblastic leukemia; CLL = chronic lymphocytic leukemia; GP = glycoprotein; NK = natural killer.

Table 11-5 Acute myeloid leukemia phenotyping.

	HLA-DR	CD34	CD33	CD13	CD11c	CD14	CD41	CD235a
M0	+	+	+	+/–	+/–	–	–	–
Ml	+	+	+	+	+/–	+/–	–	–
M2	+/–	+/–	+	+	+/–	+/–	–	–
M3	–	–	+	+	+/–	–	–	–
M4	+	+/–	+	+	+	+	–	–
M5	+	–	+	+	+	+	–	–
M6	+/–	–	–	–	+/–	–	–	+
M7	+/–	+/–	+/–	–	–	–	+	+

Table 11-6 B-lineage acute lymphoblastic leukemia phenotyping.

	TDT	CD19	CD10	CD20	Cyto-m	Surface Ig
Pro-B	+	+	–	–	–	–
Pre-Pre-B (common ALL)	+	+	+	–	–	–
Pre-B	+	+	+	1/–	+	–
Early B (Burkitt)	–	+	+	+	–	+

Cyto-m = cytoplasmic m; Ig = immunoglobulin; TDT = terminal deoxynucleotidyl transferase.

Table 11-7 T-lineage acute lymphoblastic leukemia phenotyping.

Surface	TDT	CD7	CD2	CD5	CD1	CD3	CyCD3	CD4/CD8
Prothymocyte	+	+	+	–	–	–	+	–/–
Immature thymocyte	+	+	+	+	–	–	+	–/–
Common thymocyte	+	+	+	+	+	+ /–	+	+/ +
Mature thymocyte	–	+	+	+	–	+	+	CD4 or CD8[1]
Mature T cell	–	+	+	+	–	+	+	CD4 or CD8[1]

Cy CD3 = cytoplasmic CD3; TDT = terminal deoxynucleotidyl transferase.

Table 11-8 Common B-cell neoplasms.

	CD20	CD5	CD10	CD23	CD43	CIg	SIg	Cyclin D1	Other
CLL/SLL	+	++	–	+ +	+ +	5%+	+	–	FMC7^{-}, CD79b^{+}
LPL	++	–	–	–	+/–	+	+	–	
PLL	++	+/–		–			++	–	
HCL	++	–	–	–	–	–	+	+/–	CD11c^{+}, CD25^{+}, CD103^{+}
MCL	++	++	–	–	++	–	++	++	FMC7^{+}
MZL	++	–	–	–	+/–	+/–	++	–	
FCL	++	–	60% +	–	–	–	++	–	BCL2^{+}
LCL	++	10%+	25%-50%+	+	+/–	+/–	+/]	–	BCL2^{+} in 30%-40%
Burkitt	++	–	+	–	–	+	+	–	
Myeloma	–	–	Occ +	–	+	++	–	15%-20%+	CD56^{+}, CD38^{+}, CD138^{+}

CIg = cytoplasmic immunoglobulin; CLL = chronic lymphocytic leukemia; FCL = follicular center cell lymphoma; HCL = hairy cell leukemia; LCL = large-cell lymphoma; LPL = lymphoplasmacytic lymphoma; MCL = mantle cell lymphoma; MZL = marginal zone lymphoma; PLL = B-cell prolymphocytic leukemia; SIg = surface immunoglobulin; SLL = small lymphocytic lymphoma.

Table 11-9 Common mature T-cell and NK-cell neoplasms.

	CD3S	CD3C	CD5	CD7	CD4	CD8	CD30	CD16	CD56	EBV
T-PLL	+	+	–	+	4.8	4.8	–	–	+	–
T-LGL	+	+	–	+	–	+	–	+	–	–
NK-leukemia	–	–	–	+/–	–	+/–	–	–	+	+
EN-NK/T	–	+	–	+/–	–	–	–	+	+	+
HS-gd lym	+	+	-	+	–	–	–	+	+/–	–
Ent-T lym	+	+	+	+	–	+/–	+/–	–	–	–
SC pannic T lym	+	+	+	+	–	+	+/–	–	–	–
PTCL-NOS	+	–	+/–	+/–	+/–	+/–	+/–	–	+	+/–
AILD	+	+	+	+	+/–	–	–	–	+	+/–
ALCL	+	–	+/–	+/–	+/–	+/–	+	–	–	–

AILD = angioimmunoblastic lymphadenopathy; ALCL = anaplastic large-cell lymphoma; CD3C = cytoplasmic CD3; CD3S = surface CD3; EBV = Epstein-Barr virus; Ent-T lym = enteropathic T-cell lymphoma; EN-NK/T = extranodal natural killer/T-cell lymphoma; HS-gd lym = hepatosplenic gamma delta lymphoma; NK-leukemia = natural killer cell leukemia; PTCL-NOS = peripheral T-cell lymphoma, not otherwise specified; SC pannic T lym = subcutaneous panniculitis T-cell lymphoma; T-LGL = T-large granular lymphocyte leukemia; T-PLL = T-prolymphocytic leukemia.

Table 11-10 Immunohistochemical diagnosis of Hodgkin disease.

	CD45	CD30	CD15	CD20	CD3	Pax5
Hodgkin (R-S cells)	–	+	+	LPHD(+)	–	weak+
B-lymphoma	+	+/–	–	+	–	+
T-lymphoma	+	+/–	+/–	–	+	–

LPHD = lymphocyte-predominant Hodgkin disease; R-S = Reed-Sternberg.

Cytogenetically, a minimum of two mitotic cells with gain of the same chromosome or with the same structural abnormality or three mitotic cells with loss of the same chromosome define a clone.

Constitutional chromosome abnormalities, associated with either congenital genetic syndromes or normal variants, are determined on peripheral blood T-lymphocytes grown in culture with phytohemagglutinin (PHA), a T-cell mitogen.

Fluorescence in situ hybridization (FISH) is a cytogenetic technique that uses specific fluorescently labeled DNA probes to identify each chromosomal segment. FISH can be performed using either cultured or uncultured preparations. In the uncultured technique, the assay is performed using nuclear DNA from interphase cells that are affixed to a microscope slide. FISH can be performed using bone marrow or peripheral blood smears or fixed and sectioned tissues.

Hybridization of centromere-specific probes is used to detect monosomy, trisomy, and other aneuploidies. Chromosome-specific libraries, which paint the chromosomes, are useful in identifying marker chromosomes or structural rearrangements, such as translocations. Translocations and deletions also can be identified in interphase or metaphase by using genomic probes that are derived from the breakpoints of recurring translocations or within the deleted segment. Multiplex FISH (spectral karyotyping) consists of simultaneously painting all chromosomes in a cell using different colors for each chromosome.

Cytogenetics is particularly useful in the subclassification of acute myeloid leukemias and in confirming the diagnosis and prognosis of B-cell neoplasias. CLL, acute leukemias, B-cell lymphomas, and multiple myeloma all have cytogenetic abnormalities that can be detected using either conventional cytogenetics or FISH.

Molecular diagnostics

Polymerase chain reaction

PCR is designed to permit selective amplification of a specific target DNA sequence within total genomic DNA or a complex complementary DNA (cDNA) population. Partial DNA sequence information from the target sequences is required. Two oligonucleotide primers, which are specific for the target sequence, are used. The primers are added to denatured single-stranded DNA. A heat-stable DNA polymerase and the four deoxynucleotide triphosphates are used to initiate the synthesis of new DNA strands. The newly synthesized DNA strands are used as templates for further cycles of amplification. The amplified DNA sequence can be detected by electrophoresis on an agarose gel, and visualization can be accomplished by the use of a DNA dye; alternatively, the amplified DNA can be sequenced directly in an automatic sequencer.

Uses of PCR in clinical laboratories include detection of the break cluster region-Abelson tyrosine kinase (*BCR-ABL*)

translocation in chronic myeloid leukemia, detection of promyelocytic leukemia-retinoic acid receptor alpha (*PML-RARA*) in acute promyelocytic leukemia, and detection of the Janus kinase-2 (*JAK2)* mutation in polycythemia vera. Essential and primary myelofibrosis PCR is of increasing importance in the diagnosis of acute myeloid leukemia, particularly for the detection of internal tandem duplications in the FMS-like tyrosine kinase 3 (FLT-3) locus, mutations in CCAAT/enhancer binding protein α CCAAT enhancer binding protein alpha (CEBPA), and point mutations in the nucleophosmin 1 (*NPM1*) and Wilms tumor 1 (*WT1*) genes.

DNA sequencing is important in the identification of point mutations. The earlier Sanger (chain termination) technique rapidly is being eclipsed by the next-generation sequencing technology, which has a high throughput capacity and thus makes the parallel analysis of many genes possible. The potential clinical uses, including diagnosis, predictors of response to therapy, and risk stratification are being explored for a variety of malignancies, including myeloma, leukemias, and lymphoma.

Miscellaneous laboratory hematology methods

Erythrocyte sedimentation rate

The erythrocyte sedimentation rate (ESR) measures a physical phenomenon—the opposing forces of gravity and buoyancy on RBCs when blood is suspended in an upright tube—and is expressed in millimeters per hour. Elevated plasma proteins, primarily fibrinogen and immunoglobins, neutralize red blood cell membrane negative charge, facilitating rouleaux formation and more rapid sedimentation because of increased mass per surface area. The clinical utility of ESR generally is poor except for selected rheumatologic disorders, and it is not an appropriate screening test in asymptomatic patients. Conditions associated with elevated ESR include malignancies, infections, and inflammatory conditions, particularly polymyalgia rheumatic and temporal arteritis, as well as hematologic conditions, such as cold agglutinin disease, cryoglobulinemia, and plasma cell dyscrasia–related M proteins. ESR reference ranges increase with age and are higher for women. Additional variables affect ESR; anemia and macrocytosis can cause faster sedimentation, whereas sickle cells, by impeding rouleaux formation, and microcytosis cause slower sedimentation. The modified Westergren method (EDTA blood diluted 4:1 in sodium citrate and put in a 200 mm vertical glass tube) is the preferred manual method. Automated ESR devices monitor sedimentation for shorter periods, extrapolate to millimeters per hour, and correlate reasonably well with the Westergren method.

Solubility testing for hemoglobin S

Manual qualitative methods to detect hemoglobin S (Hgb S) rely on visual detection of turbidity when blood containing Hgb S is added to a solution containing a reducing agent, detergent to lyse red blood cells, and high-concentration salt buffer. Deoxygenated Hgb S forms tactoids that defract and reflect light, whereas nonsickling hemoglobins remain soluble, allowing the detection of lines or letters when viewed through the hemolysis solution. A positive solubility test cannot discriminate between Hgb S trait, Hgb S homozygous, and Hgb S β-thalassemia. FP results can occur due to paraprotein or cryoglobulin precipitation, and FN results can occur in anemic (hemoglobin <7.0 g/dL) sickle trait individuals or when Hgb S concentration is <2.6 g/dL. Because the concentration of Hgb S in affected newborns is low, sickle solubility testing should not be performed on infants <6 months old because of the risk of FN results. If used as a screening test, a positive solubility test requires evaluation by an alternative method to confirm and quantify Hgb S and to identify coexisting nonsickling hemoglobinopathies or thalassemias. Other rare hemoglobinopathies produce a positive solubility test, including hemoglobin C Harlem, and if coinherited with Hgb S, they will produce a sickle cell disease phenotype.

Hemoglobin electrophoresis

Methods to separate normal (hemoglobins A, A_2, and F) and abnormal hemoglobins, primarily based on differences in charge, include alkaline and acid gel electrophoresis, isoelectric focusing, high-performance liquid chromatography (HPLC), and capillary electrophoresis (Figure 11-2). No method can definitively identify and quantify all hemoglobin variants, and any abnormal hemoglobin identified by the method chosen for screening must be confirmed by an alternative method (including solubility test for presumed Hgb S). HPLC and capillary electrophoresis instruments are fully automated, provide precise measurements of normal and variant hemoglobins, and are well suited for laboratories performing many analyses to diagnosis hemoglobins S, C, and E and other uncommon hemoglobinopathies and β-thalassemia trait (elevated Hgb A_2, microcytic anemia). For optimal genetic counseling, DNA analysis may be appropriate to completely characterize α-thalassemias and some uncommon thalassemias and hemoglobinopathies.

G6PD testing

Evaluation for inherited RBC enzymopathies is appropriate in patients with nonspherocytic, nonimmune-mediated hemolytic anemia. X-linked inheritance of G6PD deficiency is the most common RBC enzyme defect and is associated with hemolysis during oxidative stresses because of

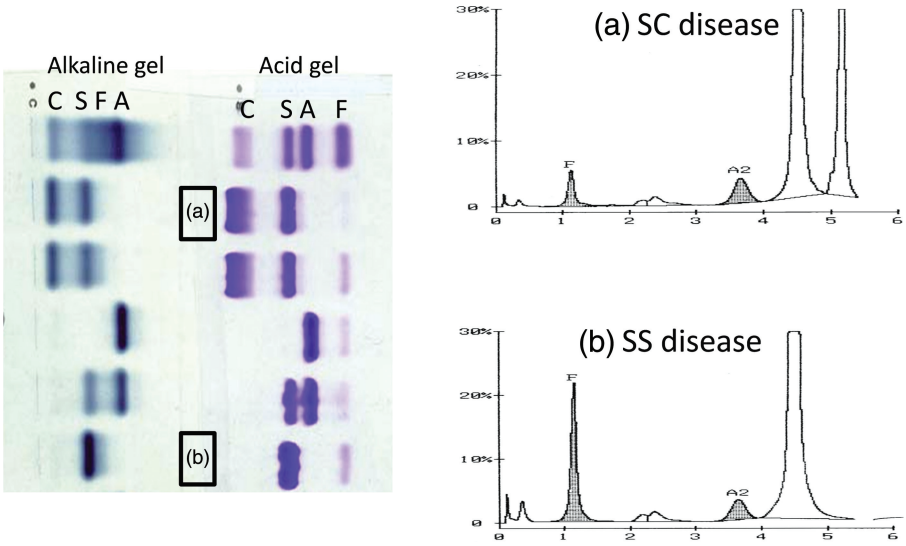

Figure 11-2 Examples of alkaline and acid gel electrophoresis and high-performance liquid chromatography patterns for (a) a patient with hemoglobin SC disease and (b) a patient with homozygous SS sickle cell disease.

acute illness, medications, or, rarely, ingestion of fava beans. Decreased G6PD activity diminishes nicotinamide adenine dinucleotide phosphate (NADPH) production and prevents reduction of methemoglobin by reduced glutathione, leading to denatured hemoglobin (Heinz bodies) and shortened RBC survival. Sensitive qualitative screening tests for G6PD deficiency include dye decolorization and fluorescent spot tests, which monitor NADPH-dependent chemical reactions. FN results may occur if testing is performed during or shortly after a hemolytic event in individuals (typically African and African American males) with the A-mutation, however, because enzyme activity is near normal in reticulocytes. Pyruvate kinase deficiency is the second most common RBC enzyme defect, presenting with chronic hemolysis of variable severity and an autosomal recessive inheritance pattern. In patients with hemolysis, a suspicion for an RBC enzymopathy, and normal G6PD screening, blood should be sent to a reference laboratory that offers a panel of additional RBC enzyme tests.

Hereditary red cell skeletal disorders

The unique flexibility of a red cell depends on its lipid bilayer attachment to an underlying scaffold of α- and β-spectrin dimers via transmembrane proteins and other linking molecules. Inherited quantitative and qualitative red cell cytoskeleton defects are an infrequent cause of nonimmune chronic hemolysis, but these defects are relatively more common in people of northern European ancestry. The most common phenotype is hereditary spherocytosis (HS), with an estimated incidence of 1 in 2,000 whites (see Chapter 7 for more structural details). The intensity of hemolysis can vary from severe anemia to a completely compensated state. In about 75% of HS cases, there is an autosomal-dominant inheritance pattern, and diagnosis can be made on the basis of family history, a negative direct antiglobulin test (DAT), anemia with reticuloctyosis, mild splenomegaly, and spherocytes on blood smear (typically <10% of red cells). In suspected cases of HS that appear to be sporadic, or if data on family members are unavailable, laboratory studies are indicated to confirm loss of red cell membrane, anchoring proteins, and spectrin. Although spherocytes are more susceptible to lysis when suspended in hypotonic saline solutions because of a decreased surface area or volume, increased osmotic fragility (OF) is an insensitive screening test for mild and compensated HS, and OF can produce FP results. A more sensitive and specific method is detection of decreased eosin-5-maleimide (EMA) binding by flow cytometry due to loss of red cell membrane proteins. Hereditary elliptocytosis causes minimal, if any, anemia and is a morphologic diagnosis (normal OF and EMA binding). Pyropoikilocytos is caused by inheritance of both qualitative and quantitative red cell skeletal defects, which produce severe hemolytic anemia, deranged red cell morphologies, and decreased EMA.

Hemostasis and thrombosis

Hemostasis involves multiple molecular and cellular interactions to initiate and regulate platelet aggregation (primary hemostasis) and coagulation (secondary hemostasis) at the site of vascular injury to produce a durable "patch" without occluding blood flow. Laboratory evaluation of hemostasis is performed in several clinical settings, including screening of asymptomatic patients before selective invasive procedures and of patients with underlying disorders associated with bleeding complications, evaluation of patients with personal or family histories of abnormal bleeding or bruising, assessment for inherited and acquired thrombosis risk factors, and antithrombotic drug monitoring.

Hemostasis screening typically consists of a prothrombin time (PT), activated partial thromboplastin time (aPTT), and platelet count. Abnormal screening test results require additional clinical and laboratory investigation to determine the etiologies. Mucosal bleeding, menorrhagia, petechiae, and ecchymoses suggest primary hemostasis disorders such as von Willebrand disease (vWD) and qualitative platelet disorders, whereas hematomas, hemarthroses, and delayed bleeding suggest a coagulation defect.

Testing for thrombophilia usually is performed when a patient has a venous thromboembolic event (VTE) in the absence of compelling acquired risk factors, such as major surgery or trauma, cancer and its treatment, and immobility. The decision to test for a predisposition to VTE also depends on the patient's age, history of thrombosis, family history of thrombosis, and whether the results would influence duration of anticoagulant therapy. Laboratory testing for inherited deficiencies of coagulation regulatory proteins should be done after a patient has completed treatment for a VTE and is in stable health. The levels of protein C (PC), protein S (PS), and antithrombin can decrease during the acute phase of a VTE. PC and PS levels are reduced by anticoagulation with warfarin. Antithrombin levels are lower during anticoagulation with unfractionated heparin. Lupus anticoagulant (LAC) testing ideally should be done before anticoagulation is begun, in conjunction with serologic assays (anticardiolipin and β-2-GPI antibodies), and abnormal results should be repeated at least 12 weeks later to determine whether they are persistent to fulfill the laboratory criteria for antiphospholipid antibody syndrome (APS). Genetic thrombophilia testing (factor V Leiden and prothrombin gene mutation 20210) can be ordered at any time and is not affected by clinical status or medications. Heparin-induced thrombocytopenia (HIT) and thrombotic thrombocytopenia (TTP) are unique acquired thrombocytopenia disorders with the potential for thrombotic complications. Laboratory test results can provide subsequent support for these diagnoses, but immediate therapeutic interventions largely should be based on clinical assessment.

Two major forms of anticoagulation therapy, warfarin antagonism of vitamin K–dependent γ-carboxylation of coagulation factors X, IX, VII, and II, and unfractionated heparin, require therapeutic drug monitoring because of unpredictable anticoagulant activities. Efforts to harmonize interlaboratory monitoring of warfarin with the PT ratio and heparin with the aPTT have led to the international normalized ratio (INR) and heparin activity (chromogenic anti-Xa) assays, respectively.

It is important to be aware of the differences in hemostasis factor reference ranges among infants, older children, and adults to avoid mislabeling newborns with inherited defects (see Chapter 2 for details). The following sections provide specific information regarding hemostasis laboratory methods as they apply to the aforementioned clinical situations.

Screening coagulation testing

Most coagulation reactions are believed to be initiated by tissue factor activation of factor VII. Important interactions occur between the extrinsic and intrinsic pathways. Although the division into two separate pathways, as shown in Figure 11-3, does not reflect complex interactions among coagulation

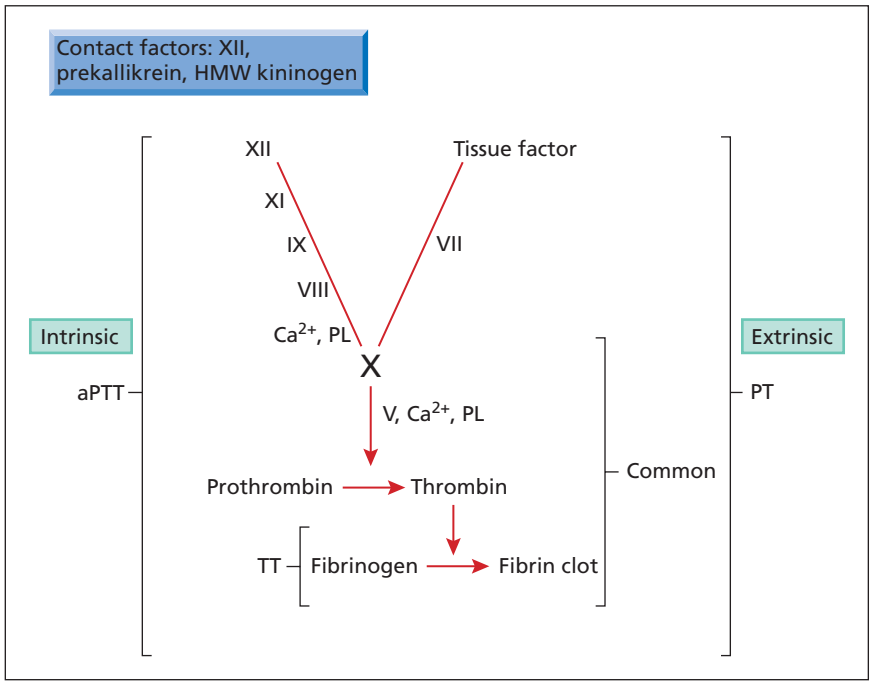

Figure 11-3 Simplified coagulation cascade indicating the intrinsic pathway measured by the activated partial thromboplastic time (aPTT), the extrinsic pathway measured by the prothrombin time (PT), the common pathway (factor X, factor V, prothrombin, and fibrinogen) measured by PT and aPTT, and the conversion of fibrinogen to fibrin measured by the thrombin time (TT). HMW = high molecular weight.

factors, it does provide a useful way to interpret screening coagulation test results when evaluating a clinical problem.

Preanalytical variables

The aPTT and, to a lesser degree, the PT are sensitive to changes in the ratio of sodium citrate solution in the collection tube and added plasma. Filling a tube with less than the recommended volume to obtain a sodium citrate-to-blood ratio of 1:9 or collecting blood in the proper proportions from a polycythemic patient increases the concentration of citrate in the plasma compartment, leading to incomplete recalcification when a fixed volume of $CaCl_2$ is added and to artifactual prolongation of the aPTT. Heparin contamination due to blood collection from central lines can cause a prolonged aPTT. A prolonged thrombin time (TT) that corrects when repeated after treatment of plasma with a heparin-neutralizing confirms heparin contamination. Alternatively, a prolonged TT and a normal Reptilase time, which uses a snake venom not neutralized by heparin-accelerated antithrombin, will confirm the presence of heparin. Most PT reagents contain heparin-neutralizing agents, making this screening clotting test insensitive to heparin contamination. Many coagulation tests performed on plasma from patients taking oral direct thrombin (dabigatran) and factor Xa (rivaroxaban) anticoagulants are at risk for either positive or negative biases, which can be clinically important (Table 11-11). The purpose of a mixing study is to determine whether a prolonged aPTT or, occasionally, a prolonged PT is more likely due to a deficiency of one or more coagulation factors or to an inhibitory antibody. The first step is to rule out contamination with heparin or direct thrombin inhibitors by performing a TT; if the TT is prolonged, the cause must be determined before proceeding. Next, the aPTT or PT is repeated on a 1:1 mixture of patient plasma and pooled normal plasma (PNP), which should provide at least 50% activity for all coagulation factors and substantial correction if deficiency is the cause. Because factor VIII inhibitors and some LACs manifest their effect in prolonging the aPTT in a time- and temperature-dependent manner, 1:1 mixtures are incubated at 37°C for 1-2 hours followed by repeating the aPTT. There is no consensus approach for interpretation of

Table 11-11 Coagulation tests interference caused by new direct oral anticoagulants.

Test	Dabigatran oral factor IIa inhibitor	Rivaroxaban and other oral factor Xa inhibitors	Comments
APCr ratio	+bias	+bias	Risk of missing FVL
Antithrombin, anti-Xa method		+bias	Risk of missing AT deficiency
Antithrombin, anti-IIa Method	+bias		Risk of missing AT deficiency
Factors X, VII, V, II (PT based)	–bias	–bias	Possible inhibitor pattern
Factors PK, HMWK, XII, XI, IX, VIII (aPTT based)	–bias	–bias	Possible inhibitor pattern
LA screen	prolonged	prolonged	
LA screen/confirm	prolonged	prolonged	Risk of false + LA
Protein C clotting Assay	+bias	+bias	Risk of missing PC deficiency
Protein S clotting Assay	+bias	+bias	Risk of missing PS deficiency
PT and aPTT	prolonged	prolonged	
PT 1:1 mix	prolonged	prolonged	Inhibitor pattern
aPTT 1:1 mix	prolonged	prolonged	Inhibitor pattern
Thrombin time	prolonged	unaffected	
Fibrinogen based on clot formation	–bias with some methods	unaffected	
Chromogenic anti-Xa Monitoring of heparin/LMWH	unaffected	positive bias	Not a quantitative test for Rivaroxaban unless calibrated with the drug

aPTT = activated partial thromboplastin time; AT = antithrombin; HMWK = high–molecular weight kininogen; LA = lupus anticoagulant; LMWH = low–molecular weight heparin; PC = protein C; PK = prekallikrein; PS = protein S.

mixing study results, and inflexible requirements such as correction to within the laboratory's PT or aPTT reference ranges to rule out inhibitor activity can be misleading. One must consider the clinical context (bleeding or thrombosis events) and the initial extent of PT and aPTT prolongation when assessing the 1:1 mix results. Sometimes mixing studies will not be definitive, especially when an aPTT is mildly prolonged and corrects with mixing, in which case performing both selected factor activity assays and LAC screening will be necessary.

Coagulation factor activity assays

Determination of a coagulation factor activity in a patient's plasma typically is performed on automated instruments and requires two reagents: PNP and plasma completely deficient in the factor of interest. Combining equal volumes of plasma from a large number of healthy adults averages the interindividual variability for coagulation factors, which typically ranges from ~50% to 150%, to produce PNP with 100% activity for all factors. Mixing PNP and factor-deficient plasma in different ratios produces calibrators of known factor activities. PTs are performed on the calibration samples for factors VII, X, V, and II, and aPTTs are performed for the intrinsic pathway factors. When the factor activities of the calibrators are plotted against the corresponding PT or aPTT results on logarithmic axes, a line or curve is generated. Then, a PT or aPTT is performed on patient plasma mixed with factor-deficient plasma, and the corresponding activity is determined from the calibration curve.

Additionally, factor levels are determined on serial dilutions of a patient's plasma, and the results, corrected for the dilution factor, are compared. If an inhibitor is present, the factor activity appears to increase with dilution. To determine whether the inhibitor interference is specific for one factor, such as factor VIII, or nonspecific like an LAC may require performance of additional factor activities.

The end point for most coagulation tests is detection of a fibrin clot. A factor VIII chromogenic activity assay exists but is not widely used. The end point of this assay is cleavage of a small peptide by an activated coagulation factor that generates a change in color (optical density) proportional to the activity of the factor. Chromogenic assays are more precise than clotting assays, but they may not detect some defects in a factor that disrupt the binding of the factor to its natural (larger) substrate.

Prothrombin time

The PT measures the time to form a fibrin clot after adding thromboplastin (tissue factor combined with phospholipid) and $CaCl_2$ to citrated plasma and assesses three of the four vitamin K–dependent factors (factors II, VII, and X) plus factor V and fibrinogen. Commercial thromboplastins contain either recombinant human tissue factor combined with phospholipid or thromboplastins derived from rabbit or bovine tissues. Almost all PT reagents contain a heparin-neutralizing additive to allow for monitoring of warfarin during concurrent heparin therapy.

Isolated prolongation of the PT most often reflects a deficiency of vitamin K–dependent factors resulting from poor nutrition, inadequate absorption of vitamin K, antagonism of γ-carboxylation of the vitamin K–dependent factors by warfarin, or decreased hepatic synthesis. Congenital deficiencies of factors X, V, and II and fibrinogen are rare (1 in 1-2 million people), whereas the estimated prevalence of homozygous factor VII deficiency is 1 in 300,000 people. Some factor VII mutations produce greater PT prolongations with rabbit or bovine tissue factor than with human tissue factor. Therefore, it is important to confirm a suspected congenital factor VII deficiency by measuring factor VII activity with recombinant human thromboplastin. Dysfibrinogenemia occasionally causes a prolongation of the PT without a prolongation of the aPTT, and factor VII inhibitory autoantibodies are extremely rare (Figure 11-4).

Warfarin causes prolonged PTs (and variably, prolonged aPTTs depending on the degree of factor IX, X, and II deficiencies). Therapeutic monitoring of warfarin depends on the PT. Thromboplastins, however, have different sensitivities to the effects of warfarin. To account for this variability, and to obtain an international sensitivity index (ISI),

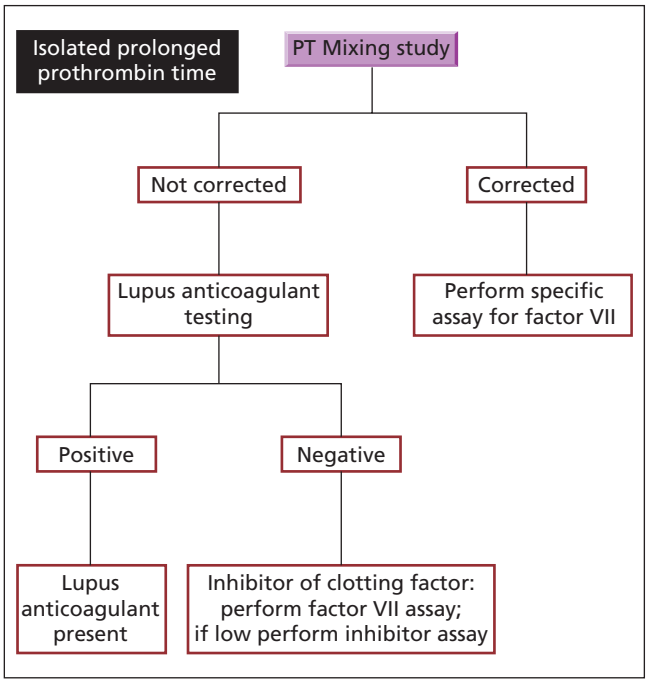

Figure 11-4 Algorithm for evaluation of an isolated prolonged prothrombin time.

manufacturers compare PTs obtained with commercial thromboplastin lots to a World Health Organization reference thromboplastin, with the behavior of human tissue factor, performed on plasma samples from healthy controls and stable anticoagulated patients. A sensitive thromboplastin with an ISI of 1.0 is equivalent to human tissue, whereas a thromboplastin with an ISI of 2.0 is relatively insensitive to depletion of vitamin K–dependent clotting factors. The INR is the ratio of the patient's PT to the laboratory's mean normal PT raised to the exponent of the thromboplastin ISI.

The reference range for an INR is typically 1.0 ± 0.2. The INR is designed to accurately monitor patients who have been stabilized on warfarin. It is *not* intended for assessing coagulopathies due to liver disease or new direct factor IIa and factor Xa inhibitors because the ISI is not validated for these conditions.

Activated partial thromboplastin time

The aPTT is a two-step assay to measure the time to form a fibrin clot after incubation of citrated plasma with phospholipid and negatively charged particles followed by the addition of $CaCl_2$. The negative surface and the phospholipid activate the contact factors (factor XII, prekallikrein [PK], and high–molecular weight kininogen [HMWK]) and factor XI. The addition of $CaCl_2$ permits activation of factor IX and the remaining reactions to proceed to form a fibrin clot.

Causes of an isolated prolonged aPTT include preanalytical artifacts, congenital factor deficiencies, acquired inhibitors, and anticoagulation therapies (Figure 11-5).

Deficiencies of factors VIII, IX, XI, XII, PK, and HMWK prolong the aPTT. Severe deficiencies of factor XII, PK, and HMWK are rare, typically produce aPTTs >100 seconds, and do not cause a bleeding disorder. Depending on the coagulation reagents and instrument in use, for an isolated intrinsic factor deficiency to prolong the aPTT, activity is usually <30%-40%.

Factors VIII and IX deficiencies, or hemophilia A and B, respectively, are X-linked inherited disorders that often are diagnosed early in life due to spontaneous bleeding or a positive maternal family history of hemophilia. Occasionally, diagnosis is delayed until adulthood if it is a mild deficiency (5%-40%).

Patients with type 1 vWD may have a slightly prolonged aPTT if the factor VIII level is low. Patients with the Normandy type 2 variant of vWD can have a moderate factor VIII deficiency.

Factor XI deficiency should be investigated when a prolonged aPTT is encountered in a person of Ashkenazi Jewish ancestry. Bleeding risk is variable and does not correlate particularly well with the severity of factor XI deficiency.

LACs can cause a prolonged aPTT (see section on thrombophilia testing). If a prolonged aPTT does not substantially shorten when repeated on a 1:1 mix with PNP, perform LAC testing or specific factor activities, depending on the clinical context.

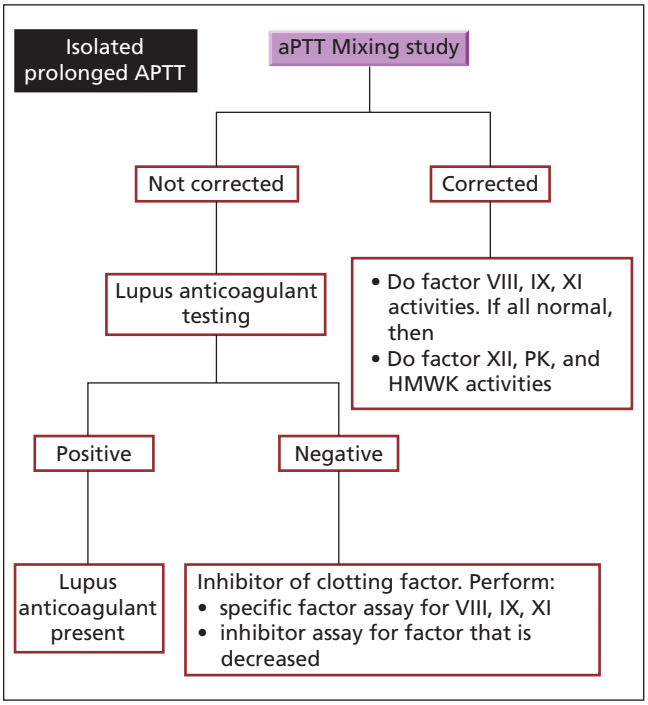

Figure 11-5 Algorithm for evaluation of an isolated prolonged activated partial thromboplastin time (aPTT). HMWK = high-molecular weight kininogen; PK = prekallikrein.

Inhibitors to factor VIII are detected in 25%-30% of males with severe hemophilia A due to the development of alloantibodies to infusions of foreign factor VIII. Alloantibody formation to factor IX in males with severe hemophilia B occurs less often. Acquired hemophilia caused by autoantibodies to factor VIII is the most common acquired specific factor inhibitor. A factor VIII antibody is suspected in patient with a prolonged aPTT that fails to fully correct immediately after 1:1 mixing and subsequently prolongs after a 1- to 2-hour incubation of the 1:1 mixture at 37°C. A very low or undetectable factor VIII activity and mild inhibitor patterns for factors IX and XI due to partial inhibition of factor VIII in these aPTT-based activity assays confirm the presence of a specific factor VIII inhibitor. The Bethesda assay determines the potency of a factor VIII inhibitor by incubating dilutions of patient plasma combined 1:1 with PNP at 37°C for 2 hours, followed by determination of residual factor VIII activity. The antibody titer is expressed in Bethesda units (BU) equal to the reciprocal of the patient plasma dilution required to obtain recovery of 50% of the expected factor VIII activity in the incubated 1:1 mixture. A BU of 0.5-5.0 is a low titer and may be overwhelmed with larger infusions of factor VIII, whereas a BU >10 will require treatment of bleeding episodes with a factor VIII bypass product, such as

recombinant factor V11a. The Nijmegen modification of the 1:1 mix conditions improves accuracy and precision of the Bethesda assay for low-titer inhibitors.

Most hospitals use aPTT-based nomograms to guide therapeutic anticoagulation with unfractionated heparin. A therapeutic aPTT range typically is determined by collecting plasma samples from patients on heparin infusions and comparing aPTTs to heparin activity using the anti-Xa chromogenic assay. The aPTT therapeutic range in seconds will correspond to an anti-Xa range of 0.3-0.7 IU/mL. The aPTT also is used to monitor the parenteral direct thrombin inhibitor argatroban, and the therapeutic target recommended by the manufacturer 1.5-3.0 times the baseline aPTT. Therapeutic infusions of direct thrombin inhibitors also prolong the PT or INR, and the intensity depends on the specific direct thrombin inhibitor and the thromboplastin reagent. The new generation of oral direct thrombin and anti-Xa inhibitors prolong the aPTT and PT (Table 11-11). They cannot be used to predict plasma concentrations, however. At the time of writing, quantitative assays to measure drug levels are not widely available.

The anti-Xa assay is a variation of a chromogenic antithrombin assay (see section on thrombophilia testing) comparing an unknown concentration of heparin in the patient plasma to a calibration curve prepared with an unfractionated heparin standard. Activated factor Xa is added to the test plasma, the rate of factor Xa neutralization by antithrombin is positively correlated with the heparin concentration, and the rate of chromogenic substrate cleavage by factor Xa is correlated inversely with the heparin concentration. Directly monitoring heparin anticoagulation with the anti-Xa assay is the preferred approach in some hospitals and is an alternative to the aPTT when unusually high rates of heparin infusion are required or when a patient's baseline aPTT is prolonged because of an LAC or deficiency of a contact activator (factor XII, PK, or HMWK). Low–molecular weight heparins (LMWHs) will minimally prolong the aPTT at therapeutic concentrations. LMWHs typically do not require monitoring. Under certain situations, however, including patients of extremely low and high weights, pregnant patients, and patients with impaired renal function, monitoring plasma LMWH activity approximately 4 hours after a subcutaneous injection using a chromogenic anti-Xa assay calibrated against an LMWH is recommended.

Combined abnormalities of PT and aPTT

Deficiency or inhibition of a factor in the common pathway (factors X, V, II, and fibrinogen), hypofibrinogenemia, dysfibrinogenemia disseminated intravascular coagulation (DIC), and lupus can cause combined prolongation of the PT and aPTT. Advanced liver disease can cause decreased hepatic synthesis of all coagulation factors, except for factor VIII, and acquired dysfibrinogenemia, which is suggested by a low fibrinogen level in a functional assay combined with a normal or high level of immunologic fibrinogen (see the section on fibrinogen assays). See Figure 11-6 for evaluation of a prolonged PT and aPTT.

Symptomatic inhibitors to factor V rarely occur after patient exposure to bovine thrombin (which also contains bovine factor V) is combined with fibrinogen to produce "fibrin glue" during surgical procedures to control bleeding. Bovine factor V antibodies may cross-react with human factor V to cause bleeding. Low–factor V activity and specific in vitro inhibition of factor V confirm the diagnosis. Fortunately,

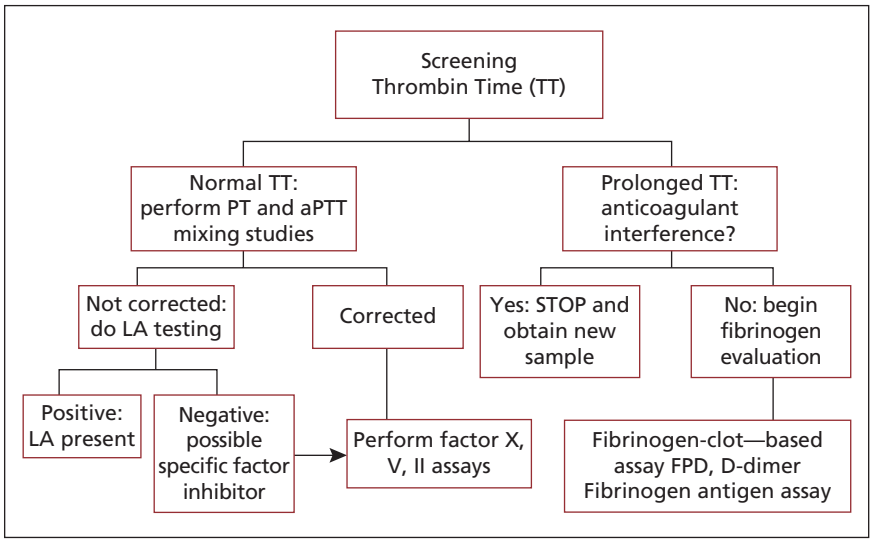

Figure 11-6 Algorithm for evaluation of a prolonged prothrombin time (PT) and activated partial thromboplastin time (aPTT). FDP = fibrin degradation product.

fibrin glue therapeutics containing either plasma-derived or recombinant human thrombin are now available.

Acquired prothrombin deficiency rarely accompanies LACs, causes moderately prolonged PTs, and can cause abnormal bleeding. The autoantibodies do not produce an inhibitor pattern in mixing studies because they are not directed against the active site of the molecule. Rather, they form immune complexes, increasing the clearance rate and lowering prothrombin activity.

Thrombin time

The TT measures the time required to convert fibrinogen to a fibrin clot, bypassing the intrinsic, extrinsic, and common pathways. TT requires sufficient amounts of normal fibrinogen and an absence of thrombin inhibitors and substances that impede fibrin polymerization. The only reagent is bovine or human thrombin, and the test sample is undiluted citrated plasma.

- Unfractionated heparin, LMWH, argatroban, bivalirudin, and dabigatran inhibit thrombin and prolong the TT.
- Dysfibrinogenemias usually prolong the TT and are suspected if the functional test (clottable fibrinogen) is disproportionately low compared with an immunologic measurement of fibrinogen.
- Hypofibrinogenemia usually prolongs the TT when levels of fibrinogen are below approximately 90 mg/dL. L-Asparaginase can cause hypofibrinogenemia by inhibiting synthesis.
- Fibrin degradation products in very high concentrations and M proteins can inhibit fibrin polymerization and prolong the TT.
- Heparin-like anticoagulants (heparan sulfates) have occurred in patients with multiple myeloma and other tumors. They prolong the TT by interacting with antithrombin in a manner similar to heparin. The Reptilase time will be normal in these patients.

Fibrinogen assays

The Clauss method is a modified TT in which fibrinogen rather than the thrombin is limiting. The time to clot formation is proportional to fibrinogen activity calibrated against a standard of known concentration and expressed as milligrams per deciliter. The thrombin concentration usually is high enough to not be affected by therapeutic concentrations of heparin but can be affected by direct thrombin inhibitors. Fibrinogen also can be measured in immunologic tests (radial immunodiffusion) to evaluate for possible dysfibrinogenemia.

Reptilase time

Reptilase is snake venom that cleaves only fibrinopeptide A from fibrinogen (in contrast to thrombin, which cleaves both fibrinopeptide A and fibrinopeptide B) and results in fibrin clot formation. This assay is prolonged by hypofibrinogenemia and most dysfibrinogenemias but is not prolonged by heparin, because the Reptilase enzyme is not inactivated by antithrombin or direct thrombin inhibitors.

Global hemostasis test

Thromboelastography involves monitoring the viscoelasticity properties of whole blood during clot initiation, contraction, and lysis. Two commercial instruments: TEG (Haemonetics, Braintree, MA) and Rotem (Durham, NC) are available in the United States. The change in viscosity of blood as it clots in a cup is transmitted through a pin immersed into the blood through a mechanical-electrical transducer, producing a tracing of clot firmness over time. Certain patterns correlate with coagulopathies, fibrinogen deficiency, thrombocytopenia, and hyperfibrinolysis. Most experience with thromboelastography has been in the liver transplantation and cardiopulmonary bypass surgery settings, where rapid point-of-care hemostasis information is used to select blood component replacement products. Modest clinical research has been done with this technology in other medical settings to evaluate patients' bleeding or thrombotic risk, but thromboelastography is not ready for general use as a diagnostic test.

von Willebrand factor assays

Endothelial cells and megakaryocytes synthesize von Willebrand factor (vWF) molecules, which undergo dimerization and subsequent linkage of dimers to form vWF multimers before secretion into blood. Once released, large multimers undergo remodeling to smaller molecules via cleavage by the protease adisintegrin and metalloprotease with thrombospondin (ADAMTS13). vWF has multiple domains with specific functions to support its two activities: adhesion to connective tissue and platelets and binding factor VIII. Although most deficiencies of vWF (vWD) are congenital, vWF deficiency also can be acquired—a condition known as the acquired von Willebrand syndrome (AvWS). AvWS often is associated with lymphoproliferative disorders, particularly monoclonal gammopathy of unknown significance (MGUS), autoimmunity, hypothyroidism, and severe aortic stenosis, as well as with left-ventricular assist devices (LVAD). Laboratory testing for suspected vWD is challenging because of the variability of personal and family bleeding histories, multiple types of vWF defects, physiologic variables affecting vWF levels, and analytical imprecision of certain vWF test methods. Repeated testing frequently is indicated to confirm abnormal results before diagnosing a patient with vWD. See Chapter 8 for additional information regarding clinical presentation, classification, and management of vWD.

Initial testing for vWD

Global tests of primary hemostasis, including bleeding time and PFA-100® (Siemens) closure times, lack both sensitivity and specificity for vWD, and aPTT is an indirect and potentially insensitive screening test for low–factor VIII activity. vWF antigen concentration (vWF:Ag), vWF-mediated agglutination of platelets (vWF:RCo) or vWF binding to collagen (vWF:collagen binding activity), and factor VIII activity measurements are sufficient initial screening tests. Reference intervals for these analytes vary based on blood type, with type O individuals having mean values approximately 25% lower than non–type O controls. Some laboratories provide blood type–specific reference intervals, whereas other laboratories provide a single reference range (with lower limits of approximately 50%) and note that asymptomatic type O individuals may have vWF antigen, activity, and factor VIII levels as low as 35%-40%. It is reasonable to consider vWF levels in the range of 30% to 50% as risk factors for mild bleeding tendency rather than an inheritable disease. Fluctuations of vWF in patients during physiologic alterations associated with acute stresses, the menstrual cycle, or pregnancy make the interpretation of theses analytes problematic, and patients may require repeat testing. Several equivalent and accurate methods can be used to quantify vWF:Ag. Measuring vWF functional activity is another matter. The most widely used method is the ristocetin cofactor assay (vWF:RCo), performed on a platelet aggregometry instrument, which assesses vWF binding to platelet GPIb/IX/V complex. Ristocetin, an antibiotic, binds to vWF, causing a change in conformation that mimics the effect of high shear stress in vivo to expose the platelet-binding domain. Control platelets bind to the modified vWF multimers, causing agglutination and increased light transmission. The vWF:RCo activity is sensitive both to quantitative deficiencies of vWF (type 1 deficiency) and to mutations causing reductions in large and medium vWF multimers or defects in platelet binding (types 2A, 2B, and 2M vWD). A vWF:RCo/vWF:Ag ratio of <0.7 supports a qualitative, or type 2, vWF defect and warrants specialized confirmatory testing (Tables 11-11 and 11-12). The vWF:RCo assay is labor intensive, and imprecise, leading to the development of alternative methods to assess adhesive activity, including binding to immobilized collagen, immobilized platelet GPIb to capture vWF, and automated immunoturbidity assays using lyophilized platelets and ristocetin. An automated immunoturbidity assay using latex particles coated with monoclonal antibodies to the vWF GPIb-binding domain compares favorably with vWF:RCo activity for detection of vWD.

Specialized testing to classify vWD

Dismissing a diagnosis of vWD or confirming a diagnosis of type 1 or type 3 vWD usually can be accomplished by reviewing vWF:Ag, vWF activity, and factor VIII activity results. vWF activity or factor VIII activity much lower than vWF:Ag, however, is an indication for more specific testing. vWD multimer analysis provides qualitative information by identifying structural abnormalities that correlate with qualitative defects in vWF adhesion (Figure 11-7). Electrophoresis of

Table 11-12 Assays for vWD classification.

vWD type	vWF Activity	vWF Antigen	RIPA	FVIII	Multimers
Type 1	↓	↓	↓	↓	Nl pattern
Type 2A	↓↓	↓	↓↓	↓	↓ Large and intermediate
Type 2B	↓↓	↓	↑↑↑	↓	↓ Large
Type 2M	↓↓	↓	↓↓	↓	Normal
Type 2N*	NI	NI	NI	↓	Normal
Type 3	↓↓↓	↓↓↓	↓↓↓	↓↓↓	Undetectable

* FVIII low;
RIPA = ristocetin-induced platelet aggregation;
vWD = von Willebrand disease; 2N = Normandy variant of vWD.

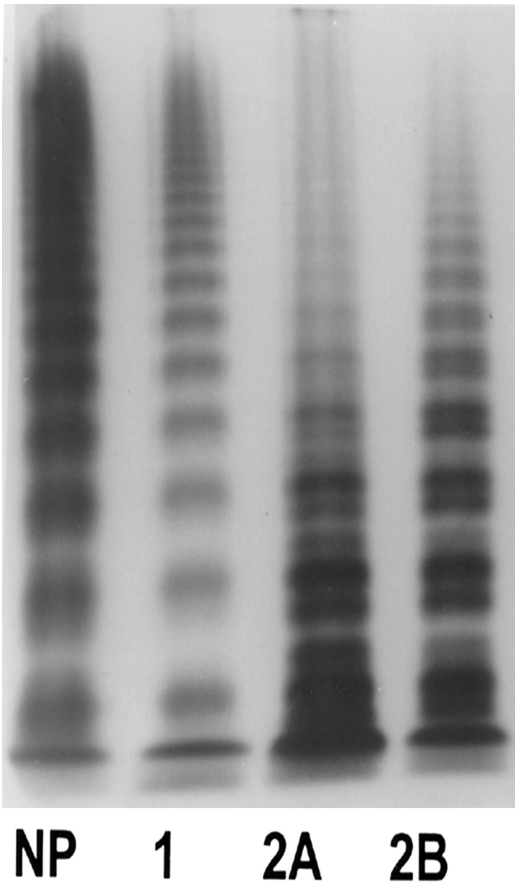

Figure 11-7 von Willebrand multimer patterns. NP = normal plasma; 1 = type 1 von Willebrand disease (vWD) with normal bands but decreased staining intensity; 2A = type 2A vWD with loss of large and intermediate multimers; 2B = type 2B vWD with loss of large multimers.

plasma through low-concentration agarose gel separates vWF multimer bands by size, which are detected with radiolabeled, enzyme-linked, or fluorescent vWF antibodies. Analysis of the band patterns can distinguish normal or subtly abnormal patterns (consistent with type 1 and 2N or 2M vWD, respectively) from major losses of large and intermediate-size bands (consistent with type 2A, type 2B, and platelet-type vWD).

The ristocetin-induced platelet aggregation assay (RIPA) is a variation on the vWF:RCo activity to investigate platelet adhesion defects. Several ristocetin concentrations (ranging from 0.6 to 1.5 mg/mL) are added to separate aliquots of a patient's platelet-rich plasma, while change in light transmission is monitored as platelets bind to vWF and aggregate (Figure 11-8). Normal and mild type 1 vWD platelet-rich plasma typically produces no or minimal aggregation at low ristocetin concentrations and increasing aggregation at higher concentrations. Platelet-rich plasma from severe type 1 and types 2A and 2M vWD patients produces attenuated aggregation at high ristocetin concentrations, whereas platelet-rich plasma from type 2B or platelet-type vWD patients shows an enhanced aggregation response to low ristocetin concentrations. Estimates of the relative frequency of type 2B vWD to platelet-type vWD range from 8-10 to 1. Although the disorders have similar clinical presentations and inheritance is autosomal dominant, they require different types of hemostasis replacement products (vWF concentrate vs. platelets, respectively). Mixing studies using normal washed platelets plus patient plasma, or vice versa, can distinguish whether the patient's vWF or platelet receptor is abnormal. Genotyping to detect known mutations associated with each disorder is offered by a few reference laboratories. Rarely, men and women with mild or moderate factor VIII deficiencies lacking X-linked inheritance pattern consistent with hemophilia A may be homozygous for type 2N vWD (decreased vWF binding affinity for factor VIII) or compound heterozygous (type 1/2N). Decreased binding of control factor VIII to the patient's immobilized vWF in an enzyme-linked immunoadsorbent assay (ELISA) and equivalent vWF:Ag and vWF activity results are consistent with type 2N vWD. Genotyping specific for type 2N mutations is offered by a few reference laboratories.

Bleeding disorders with normal screening hemostasis tests

Abnormal, typically delayed, bleeding due to severe factor XIII deficiency and fibrinolytic pathway defects is rare, yet it should be considered when evaluations for coagulopathies and primary hemostasis defects are negative. Thrombin activates factor XIII, and factor XIIIa cross-links fibrin monomers to produce a durable clot. The urea clot lysis test is a qualitative screening test for severe factor XIII deficiency. Thrombin is added to plasma, and the clotted fibrin is added to a high-molar solution of urea that will disrupt the clot if fibrin has not been cross-linked by factor XIIIa. Alternative quantitative assays are available to directly quantify factor XIII concentration and activity. Global screening tests of the fibrinolytic system include the euglobulin clot lysis time (ECLT), which measures the time to lyse a fibrin clot in the absence of plasmin inhibitors, and the whole blood clot lysis time (see thromboelastography). Congenital hyperfibrinolysis is due to deficiencies of tissue plasminogen activator (tPA) or plasmin natural inhibitors, and laboratory evaluation requires a panel of analytes, including plasminogen, plasminogen activator inhibitor 1 (PAI-1) activity, and antigen, tPA antigen, and α_2-antiplasmin activity.

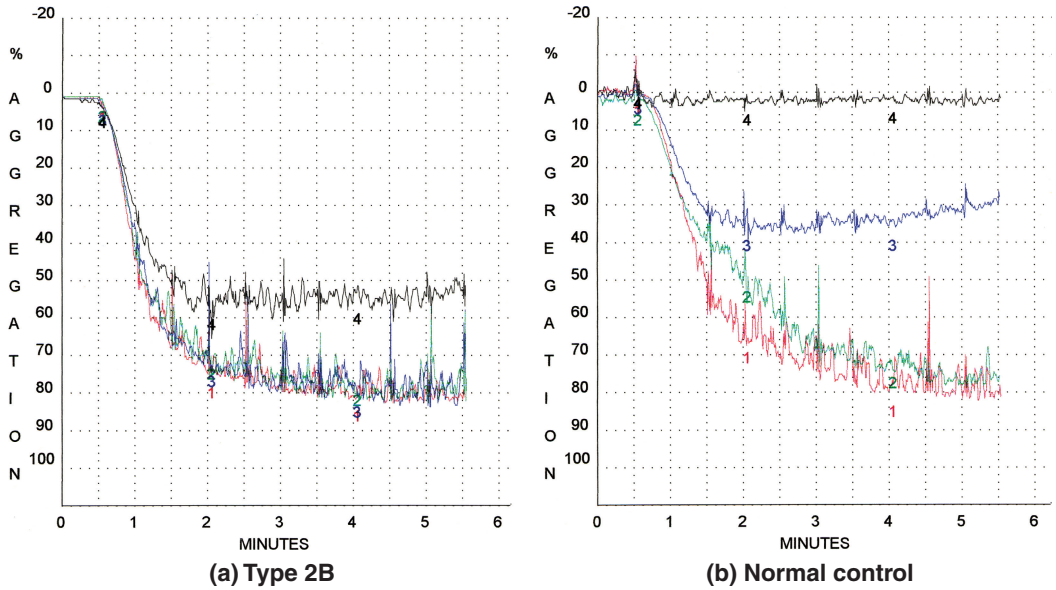

Figure 11-8 Examples of platelet-rich plasma aggregation responses to a range of ristocetin concentrations [1 = 1.5, 2 = 1.2, 3 = 0.9, 4 = 0.6 (mg/mL)]. (a) Type 2B vWD patient showing >50% aggregation with all ristocetin concentrations; (b) Normal control demonstrating concentration-dependent aggregation.

Causes of acquired hyperfibrinolysis resulting in circulating plasmin overwhelming α_2-antiplasmin inhibition include decreased hepatic clearance of tPA due to advanced cirrhosis or during liver transplantation, increased release of tPA from endothelial cells during cardiopulmonary bypass, amyloidosis, envenomization from several species of snakes, and as a component of the disseminated intravascular coagulation (DIC) process associated with acute promyelocytic leukemia and rarely with solid tumors, including prostate cancer. Laboratory support for primary fibrinolysis includes reduced fibrinogen levels due to cleavage by plasmin, elevated fibrin(ogen) degradation products, and no significant elevation of D-dimer levels because lysis of cross-linked fibrin clot is not the dominant process. DIC is the result of a primary disease process that leads to the release of tissue factor or other coagulation-activating factors into the blood (see Chapter 2 for more details). Because of variations in the amount and rate of procoagulant material released determined by the underlying disease, there are no diagnostic patterns of laboratory results. In acute, overwhelming DIC, initial platelet counts and fibrinogen levels are low, or serial testing shows a downward trend. PT, aPTT, and TT may be prolonged, depending on the severity of consumption, and D-dimer levels are markedly elevated, indicating unregulated thrombin activity and secondary fibrinolysis.

Vessel wall defects, such as collagen diseases (eg, Ehlers-Danlos and Marfan syndromes), also can cause abnormal bleeding. In addition to physical examination and imaging information, genetic testing is becoming more readily available for some of these syndromes.

Platelet function tests

In vitro assessment of platelet activation and aggregation in response to selected platelet agonists should be reserved for patients with convincing bleeding histories in whom evaluations for coagulopathies, vWD, and moderate to severe thrombocytopenia are negative. In addition, prescribed and over-the-counter medications that can inhibit platelet function must be discontinued before testing. Many disease processes can produce acquired qualitative platelet defects, including uremia, liver failure, and myeloproliferative and myelodysplastic disorders, but formal aggregation studies testing usually are not informative in these cases. Platelet function testing is technically demanding, time consuming, and poorly standardized, although efforts are under way to develop guidelines for performing and interpreting these studies. The hematologist should be aware that labs use two different platforms to analyze platelet aggregation: instruments that are used to test platelet-rich plasma and instruments that use whole blood samples (whole blood aggregometry [WBA]). Testing is performed on aliquots of citrated blood or platelet-rich plasma with different concentrations of agonists, such as adenosine diphosphate (ADP), epinephrine, and collagen; arachidonic acid, which platelets metabolize to the agonist thromboxane A_2 via the cyclooxygenase pathway; and ristocetin to screen for platelet GPIb/IX/V deficiency. Formation of platelet aggregates causes an increase in light transmission over time. Figure 11-9 shows a normal aggregation response of platelet-rich plasma to collagen and ADP, and a clear first and second wave with epinephrine, indicating initial aggregation in response to exogenous epinephrine followed by additional, irreversible aggregation because of a release of ADP from platelet-dense granules. The platelet release reaction can be assessed in a lumi-aggregometer, which simultaneously monitors WBA through changes in electrical impedance as platelets aggregate and platelet activation when released adenosine triphosphate combines with luciferin-luciferase enzyme-releasing light. Certain patterns of platelet aggregation responses to a panel of agonists are sensitive to specific inherited and rare qualitative platelet disorders, including Glanzmann thrombocythemia, Bernard-Soulier disease, and collagen receptor defects. Platelet secretion defects resulting from abnormal signal transduction and qualitative and quantitative granule disorders are more common, produce variable aggregation patterns, and require additional diagnostic tests that are not readily available for clinical use, but these tests may be accessible through research laboratories.

Global primary hemostasis screening tests

The template bleeding time is an invasive test, fraught with difficult-to-control technical and patient variables, that lacks specificity and sensitivity for detection of primary hemostasis disorders. Prolonged bleeding times performed on asymptomatic patients do not predict a risk of abnormal bleeding during surgery or other invasive procedures. The test is performed by making a standard incision in the forearm using a spring-loaded blade while maintaining a blood pressure cuff at 40 mm Hg. Blood oozing from the incision is wicked away with filter paper every 30 seconds until bleeding stops. The typical reference range in adults is approximately 5-10 minutes.

Most laboratories have discontinued performing template bleeding times and substituted automated in vitro screening methods, which do not require an incision and provide precise results from samples of blood collected in citrate, yet have similar limitations. The PFA-100 instrument monitors vWF-dependent platelet adhesion and aggregation under conditions that mimic the shear forces in the arterial circulation. Citrated blood is aspirated through a minute hole in a membrane coated with collagen and ADP (COLL/ADP) or collagen and epinephrine (COLL/EPI). vWF multimers bind

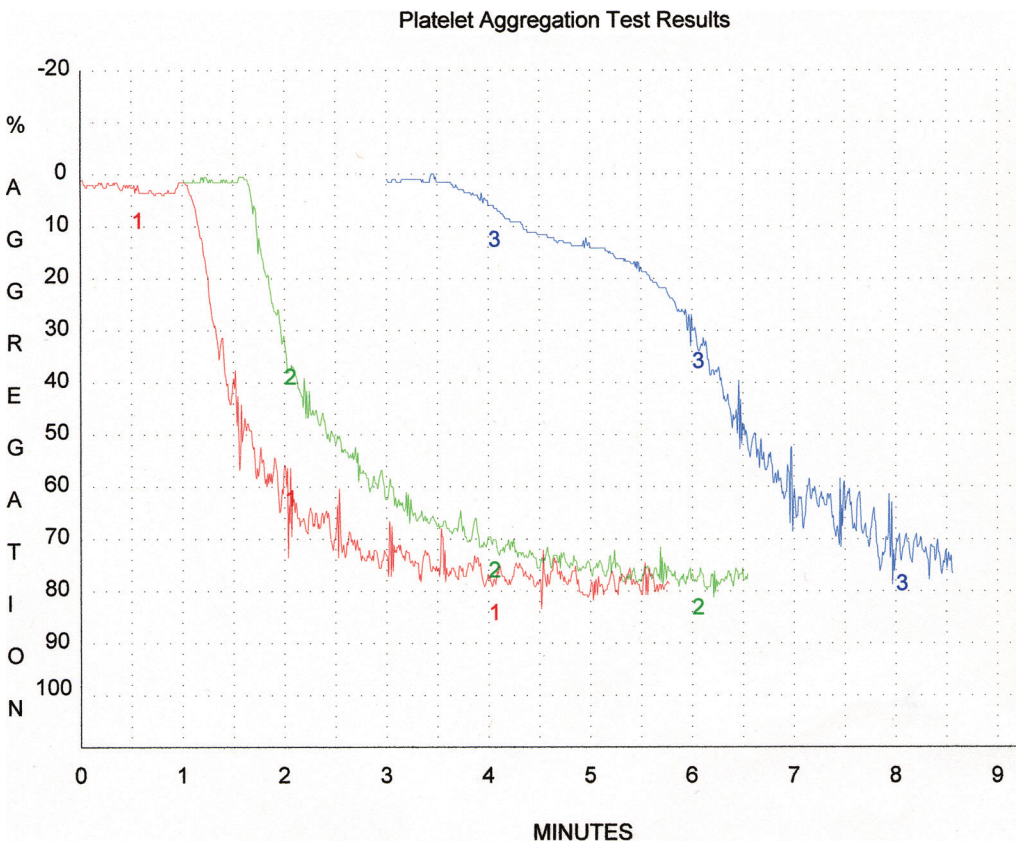

Figure 11-9 Representative platelet aggregation curves performed on normal platelet-rich plasma. 1 = collagen 5 µg/mL, 2 = ADP 5 µg/mL, 3 = epinepherine 5 µM/mL.

to collagen and platelets adhere to vWF, are activated by COLL/ADP or COLL/EPI, aggregate, and occlude the aperture, which is recorded as closure time in seconds. Each laboratory must determine reference intervals, although typical ranges are 55-137 seconds and 78-199 seconds for COLL/ADP and COLL/EPI cartridges, respectively. Prolonged PFA-100 closure time is not sufficiently sensitive for all congenital qualitative platelet disorders and types of vWD to be used as a general screening test. In addition, as anemia and thrombocytopenia worsen, closure times increase, and these variables should be considered when interpreting prolonged closure times in the setting of hematocrit <30% and platelet count $<100 \times 10^6/\mu L$. Prolonged COLL/EPI closure time is a sensitive test for aspirin inhibition of platelets, but the COLL/ADP closure time is insensitive to blockade of the platelet P2Y12 ADP receptor by thienopyridines.

Specialized testing for acquired thrombocytopenia

Assays for platelet antibodies

Immune-mediated thrombocytopenia remains a clinical diagnosis of exclusion due to the general poor performance of laboratory methods to detect platelet-specific antibodies. Assays detecting total or surface-bound platelet immunoglobulins are nonspecific and are not recommended.

Assays for HIT

HIT is a clinical diagnosis supported by serologic and functional assays. In vitro functional assays monitor activation of normal control platelets by patient serum in the presence of therapeutic concentrations of heparin and at high heparin concentrations. Activation with a low heparin concentration and no activation at high heparin concentration are considered to be both specific and sensitive for detection of platelet factor-4 (PF4) heparin-immune complexes, which are capable of causing in vivo platelet activation, thrombocytopenia, and thrombosis. In North America, selective laboratories perform the serotonin release assay to monitor carbon^{14}–labeled serotonin secretion from control platelets. In Europe, heparin-induced platelet aggregation performed in microtiter wells with visual detection of platelet aggregation is the preferred method. Both assays are technically difficult, labor intensive, and not readily available.

Commercial ELISA assays detect antibodies recognizing immobilized PF4 bound to heparin or polyvinylsulfonate complex. Although sensitive, HIT ELISA results are nonspecific, detecting antibodies incapable of activating platelets in vitro or causing thrombocytopenia and thrombosis in vivo. The positive predictive value of a positive PF4 ELISA result alone to confirm a diagnosis of HIT is a low, and if used as the only criterion, a positive PF4 ELISA results in the overdiagnosis of HIT. Growing evidence supports three approaches to

improving the specificity of PF4 ELISA testing. First, clinicians can improve the pretest likelihood that thrombocytopenia is due to HIT by applying a validated clinical scoring system called the 4Ts (*t*hrombocytopenia, *t*iming, *t*hrombosis, and o*t*her more likely causes of thrombocytopenia). Patients with low 4T scores are unlikely to have HIT, even with a positive PF4 ELISA, removing the need for testing. This is especially true for patients who have an increased likelihood of having a FP test, such as patients who have had a cardiopulmonary bypass procedure. Second, identifying only IgG instead of a combination of IgG/IgM/IgA PF4 antibodies improves the specificity of a positive PF4 ELISA with a slight impact on sensitivity. Finally, ample evidence suggests that the higher a PF4 ELISA optical density (OD) is, the more likely a functional HIT assay will be positive and the clinical presentation and course will be consistent with HIT. No cutoff point, however, completely segregates all platelet-activating antibodies from nonactivating antibodies. Conversion from viewing PF4 ELISA results as simply positive or negative to considering OD as a continuous variable, with increasing probability for HIT as OD increases, is still evolving as clinical research continues.

Assays for TTP and vWF-cleaving protease (ADAMTS13)

In sporadic cases of TTP, ultralarge forms of vWF initiate the formation of platelet aggregates and lead to thrombi and thrombocytopenia. In these cases, the activity of the vWF-cleaving protease, ADAMTS13, typically is low (ie, <5%-10%), and in many cases, in vitro evidence of an inhibitory autoantibody is present. In hereditary forms of TTP, there are mutations in the gene encoding the enzyme, and the activity of ADAMTS13 is absent or markedly decreased; however, no inhibitor is present.

The main methods that currently are used employ a recombinant 73–amino acid peptide from the A2 domain of vWF containing the Y1605-M1606 bond recognized by ADAMTS13 to detect substrate cleavage by either ELISA or fluorescence resonance energy transfer (FRET) methods. Two amino acids in the peptide substrate are modified in the FRET assay; one fluoresces when excited, and the other absorbs or quenches the released energy. When ADAMTS13 cleaves the substrate and separates the modified amino acids, emitted energy is detected in a fluorescent plate reader. The method for ADAMTS13 neutralizing antibody detection is similar to the Bethesda assay for factor VIII inhibitors; dilutions of patient serum and plasma are mixed with PNP followed by measurement of residual enzyme activity using the synthetic substrate. Typical reference values are ADAMTS13 activity >67% and inhibitor titer <0.4. Measuring ADAMTS13 antigen is not necessary when evaluating a patient for sporadic or idiopathic TTP.

The decision about whether to initiate plasma exchange is made on the basis of clinical assessment and should not be delayed until ADAMTS13 activity and inhibitor results return because they improve diagnostic specificity at the expense of sensitivity. Persistently low ADAMST13 activity and positive inhibitor titer are predictors of relapse during remission.

Assays for thrombophilia

Inherited deficiency of one or more of the identified natural inhibitors of coagulation (antithrombin, PC, and PS) is a risk factor for venous thrombosis, and functional and immunologic assays are available to measure these inhibitors. The use of these assays generally should be restricted to patients in whom the result may affect prognosis and duration of anticoagulant treatment. This generally includes patients who present with spontaneous thrombosis not temporally related to recent surgery, trauma, immobilization, cancer, or other acquired risk factors. The likelihood of identifying a deficiency is increased if thrombosis is recurrent or in an unusual location, the patient is young (<45 years old), or the patient has a positive family history of thrombosis. To avoid misleading low results due to temporary conditions related to acute illness, thrombosis, and anticoagulation therapy, testing for nonmolecular assays ideally should be delayed until several weeks after completion of treatment when a patient has returned to baseline. The biologic and analytical variability associated with phenotypic diagnoses of these deficiencies requires verification of an abnormal test result on a new sample. Because of the large number of mutations associated with deficiencies of antithrombin, PC, and PS, genotyping is not routinely performed.

Antithrombin deficiency

The most sensitive screening tests for antithrombin deficiency are chromogenic activity assays designed to quantify antithrombin inhibition of factor Xa or IIa in the presence of unfractionated heparin. Abnormal low-antithrombin activity results require measurement of antithrombin antigen to classify the deficiency as type I (activity = antigen) or type II (activity < antigen). Type I antithrombin deficiency is more common than type II deficiency in symptomatic kindreds. Subclassification of type II deficiency requires performance of the chromogenic activity assay without heparin to differentiate type IIa resulting from reactive site defects and IIb resulting from antithrombin heparin-binding defects. Although type IIb is associated with a low risk of thrombosis, progressive antithrombin activity assays are not readily available and typically are not performed.

PC deficiency

The preferred screening tests for PC deficiency are chromogenic assays. PC is activated with a snake venom. PC activity correlates with hydrolysis of a synthetic peptide and change in OD. Clot-based PC activity assays are an alternative, but potentially inaccurate results may occur due to variations in factor VIII and PS levels, factor V Leiden, inhibitory antibodies, and anticoagulants. An abnormal low-PC activity result requires measurement of the PC antigen to classify the deficiency as type I (activity = antigen) or type II (activity < antigen).

PS deficiency

PS assays are challenging because of the unique biology of PS. Total plasma PS is partitioned between free and bound forms. The protein is nonfunctional when bound to complement 4b binding protein and functional when it is free. In its unbound form, the protein can serve as a cofactor for activated PC (aPC). The typical PS bound-to-free ratio of 60:40 varies under different physiologic and pathologic conditions. Clot-based PS activity assays are the most sensitive screening tests for PS deficiency but suffer from potential inaccuracy because of the same variables that can affect PC activity testing. An alternative screening assay is free PS antigen concentration to avoid confounding variables. Free PS testing, however, is insensitive to type II PS deficiency (low activity but normal free antigen level). Some laboratories screen with PS activity, some screen with free PS antigen, and other laboratories use both assays.

Factor V Leiden and prothrombin gene mutation

Two autosomal inherited coagulation factor variants increase the risk for VTEs; these are factor V G1691A (factor V Leiden) and prothrombin G20210A. Several sensitive commercial clot-based screening assays for factor V Leiden mutation demonstrate a resistance of factor Va cleavage by aPC in the presence of factor V Leiden mutation. Coagulation testing, activated with aPTT, PT, or Russell viper venom reagents, is performed with or without added aPC, and the clotting times are expressed as a ratio. Abnormally low ratios represent aPC resistance (aPCr). Specificity is improved by repeat testing of positive plasmas after dilution with factor V–depleted plasma to minimize impact of inhibitors, anticoagulants, and high factor VIII levels. Genotyping should be performed on all aPCr-positive patients determine whether they are heterozygous or homozygous for factor V Leiden. Although prothrombin G20210A mutation is associated with elevated prothrombin levels, measuring factor II activity is not a sensitive screening test, and genetic testing is the primary method.

Antiphospholipid syndrome

The APS is an important acquired thrombotic condition. Consensus-based criteria have been developed for the investigational diagnosis of APS. These criteria require a combination of clinical conditions (unexplained venous or arterial thromboembolic events, pregnancy complications) and persistent laboratory evidence of autoantibodies that recognize epitopes on selected proteins associated with phospholipids and identified by coagulation-based (LACs) or serologic-based (cardiolipin and β_2GPI antibodies) testing. LACs are heterogeneous antibodies that interfere with in vitro clotting assays. Indirect evidence for the presence of a LAC requires: (i) prolongation of a screening clotting assay designed to be sensitive to the phospholipid-dependent behavior of LAC, (ii) ruling out prolongation due to a coagulopathy by showing incomplete correction in a 1:1 mix of patient and normal pooled plasma, and (iii) confirming phospholipid dependence by shortening the clotting time with the addition of more phospholipid. Although some LACs are discovered when a routine aPTT is prolonged, a normal aPTT is generally not a sensitive LAC screening test and should not prevent performance of more sensitive LAC testing based on the clinical circumstances. There is no gold-standard LAC method. Recent updated consensus expert guidelines from the International Society of Thrombosis and Hemostasis Scientific Subcommittee on Lupus Anticoagulant/Phospholipid Antibodies recommend performing two sensitive LAC tests in parallel—one aPTT-based test and one Russell viper venom (activation of factor Xa)–based test—and accepting a positive result from either or both as evidence of an LAC. Preanalytical variables requiring attention include platelet contamination (>10,000/μL) due to inadequate centrifugation, which can produce FN LAC results because of the neutralizing effect of platelet-derived phospholipid, and concurrent anticoagulation therapy. The presence of a direct thrombin inhibitor or factor Xa inhibitor in the test plasma nullifies the validity of LAC testing. Heparin can be neutralized by additives in the LAC test reagents or in a separate step before testing, and the mixing step can compensate for mild to moderate coagulopathies due to liver disease or vitamin K antagonists like warfarin. The preferred time, however, for LAC testing is before or after anticoagulation treatment. Rarely, a specific factor inhibitor can cause an FP LAC result, typically with an aPTT-based LAC test due to a factor VIII inhibitor. A more frequent occurrence, however, is the appearance of multiple coagulation factor deficiencies when the true coagulation factor levels are within normal limits; this misleading picture occurs because the same antibodies responsible for the LAC effect also interfere with coagulation factor assays. The hematologist should be aware that rare patients concurrently may have both an LAC and a true factor VIII inhibitor. Abnormal bleeding likely would be

present, and specific factor assays would confirm an isolated factor deficiency. LAC tests are either positive or negative, and evidence is insufficient to support reporting gradations of positive results. Because of differences in test methods, reagents, instrumentation, preanalytical variables, and approaches to analyzing and reporting results, there is substantial interlaboratory variability of LAC results based on external proficiency testing surveys.

LAC can cause reagent-dependent prolongations of PT results. Although this is usually mild, occasionally LAC-positive patients will have elevated INRs before starting warfarin. Chromogenic factor X activity is an alternative to the INR for therapeutic anticoagulation monitoring (target 20%-40%); however, availability of the test is limited. Another option is to measure PT-based factor II, VII, and X activities and observe whether the LAC produces an inhibitor pattern on the serial dilutions of plasma. If one or more factor assays appear unaffected by the LAC, then suppression of a specific clotting factor can serve as the therapeutic target for warfarin anticoagulation. A markedly prolonged PT in the setting of LAC may be a result of acquired factor II deficiency due to a nonneutralizing prothrombin autoantibody that increases the clearance rate. These patients are at risk for spontaneous bleeding. To recognize this rare condition, a factor II activity level should be obtained in an LAC-positive patient with a prolonged PT/INR.

Performance of ELISA testing for anticardiolipin (aCL) and anti–β2GPI (aβ2GPI) antibodies should accompany LAC testing to maximize sensitivity because persistently positive (arbitrarily defined as >12 weeks apart) results from serologic tests or LAC, or both, fulfill the laboratory criteria for APS. Commercial ELISA kits for aCL and aβ2GPI lack standardization, and interlaboratory agreement is poor for weakly positive sera. To improve specificity, some experts consider only medium and high titer-positive IgG and IgM aCL and aβ2GPI results to be clinically important.

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