A CONCISE REVIEW OF CLINICAL LABORATORY SCIENCE
SECOND EDITION
This book is dedicated to clinical laboratory science students everywhere. In your upcoming role as professionals, remember that your job is important to the medical world as well as to the individual patient. Be proud of the fact that you will be making a difference in people’s lives. Always be excited about the unlimited opportunities available in your chosen profession and help lead the field of Clinical Laboratory Science well into the 21st century.
The arrival of the second edition of *A Concise Review of Clinical Laboratory Science* has long been anticipated by students and educators alike. This review text is a valuable educational tool for both the novice and the experienced clinical laboratory scientist. It is designed to be an updated and concise review of all disciplines of clinical laboratory science and will also serve as a tool for students of clinical laboratory science studying for national certification examinations, including the American Society of Clinical Pathologists Board of Registry exam, the National Certification Agency (NCA) exam, and the American Medical Technologist (AMT) exam. Practicing clinical laboratory scientists and medical residents will also find this book to be an excellent source for review.

This book represents a culmination of the efforts and expertise of the faculty of the Clinical Laboratory Science program at Texas Tech University Health Sciences Center in Lubbock, Texas, and reflects over 100 years of combined medical technology experience. All contributing authors reflect their professional excellence in their contributed chapters, not only as educators, but also as outstanding professionals in their field. I encourage readers to send me feedback on this book at the following email address: joel.hubbard@ttuhsc.edu.

**Text Format and Features**

Each chapter presents a concise summary of the most important facts and concepts in that subject area in an outline format. Key points appear in bold for easy reference. Boxes, tables, and figures throughout distill concepts and make them easier to comprehend. Online menus at the end of each chapter point readers to supplementary Web-based materials.

**What’s New in This Edition**

The second edition includes the most current and updated information. An expanded chapter dealing with laboratory operations (Chapter 11) addresses topics such as management and organizational theory, professionalism, quality assurance, laboratory regulations, and delivery of an educational unit. In addition, a new chapter on molecular pathology (Chapter 10) focuses on molecular laboratory methods and an overview on the testing of genetic diseases.

**Additional Resources**

*A Concise Review of Clinical Laboratory Science, second edition*, includes additional resources for both instructors and students that are available on the book’s companion Web site at thePoint.lww.com/Hubbard2e.
Preface

Instructors
Approved adopting instructors will be given access to the following additional resources:

- Image Bank (including color images referenced in the text)
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Students
Students who have purchased *A Concise Review of Clinical Laboratory Science, second edition*, have access to the following additional resources:

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In addition, purchasers of the text can access the searchable Full Text Online by going to the *A Concise Review of Clinical Laboratory Science, second edition*, Web site at thePoint.lww.com/Hubbard2e. See the inside front cover of this text for more details, including the pass code you will need to gain access to the Web site.
I would like to thank all of the following contributing authors—Dr. Lynne Hamilton, Dr. Hal Larsen, Dr. Barbara Sawyer, Mr. Wade Redman, Ms. Lori Rice-Spearman, and Dr. Tootie Tatum—for making this book possible. Their individual expertise, willingness to present the highest quality of material, and high level of professionalism made the task of producing this text easy. I would also like to thank my wife, Kathy, who patiently listened to my endless rambling about the project.
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CHAPTER 1
Clinical Chemistry

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I. CLINICAL CHEMISTRY BASICS

A. Laboratory math and statistical concepts

1. Concentration. Solutions can be described in terms of the concentration of the components of the solution.

a. A percent solution can be described as:
   (1) w/w, which is expressed as weight (mass) per 100 units of weight (g/g).
   (2) w/v, which is expressed as weight (mass) per 100 units of volume (g/dL).
   (3) v/v, which is expressed as volume (mL) per unit of volume (mL).

b. Molarity (M) is expressed as moles per liter (mol/L) or millimoles per milliliter (mmol/mL).
   (1) A mole is one formula weight, in grams, of a compound. For example, one mole of NaOH equals 40 g, because one molecule of sodium equals 23 g, one molecule of oxygen equals 16 g, and one molecule of hydrogen equals 1 g.
   (2) Molarity is calculated by determining what units are given in the problem, then determining the final units needed, and setting up an equation (Boxes 1–1 and 1–2).
   (3) A simple calculation for molarity problems can be performed with the following formula:

\[
\frac{\text{Grams in solution}}{\text{Volume in liters}} = \text{Formula weight} \times \text{molarity}
\]

Using the information from the first problem, the variables can be plugged in:

\[
\frac{40 \text{ g}}{1 \text{ L}} = 40 \text{ g} \times 2 \text{ M}
\]

Using the information given in the second problem, the variables can be plugged in:

\[
\frac{32 \text{ g}}{0.3 \text{ L}} = 36.5 \text{ (M)}
\]

\[
\frac{106.7}{36.5} = M
\]

\[
2.9 = M
\]
Box 1–1 Molarity

PROBLEM:
How many grams of NaOH are needed to make 1 L of 2 M solution?

ANSWER:
Final units needed: g/L
Units of measure given: M, L
By definition, a molar solution is the number of moles per liter of solution. For a 2 M solution:

\[
\frac{40 \text{ g NaOH}}{1 \text{ mole}} \times \frac{2 \text{ mole}}{1 \text{ L}} = 80 \text{ g/L} = 2 \text{ M NaOH}
\]

Eighty grams NaOH are required. To prepare the solution, 80 g NaOH are placed in a 1 L volumetric flask, and deionized water is added to make a volume of 1 L.

c. Normality (N) is expressed as equivalent weight (Eq wt) per liter of volume (Eq/L or mEq/mL).

d. Dilutions are solutions formed by making a less concentrated solution from a concentrated solution. They are stated as a part (concentrate) of the concentrated substance used plus the volume of diluent used.

EXAMPLE: 100 μl of serum in 400 μl of saline = 100 in a total of 100 + 400 = 100/500 = 1:5 dilution.

2. Hydration is the process of adding water molecules to the chemical structure of a compound. It is important to consider the molecular weight of these molecules when making solutions (Box 1–3).

B. Statistical concepts. Statistics is the science of gathering, analyzing, interpreting, and presenting data. A statistic is a number summarizing data.

1. Descriptive statistics are data that can be described by their location and dispersion compared with the average. After data are plotted on a histogram, the values typically form a symmetric curve referred to as normal or gaussian distribution (Figure 1–1).

a. The mean (x) is the arithmetic average of a set of data calculated as follows:

\[
x = \frac{x_1 + x_2 + x_3 + \cdots + x_n}{n}
\]

where \(x\) is each individual value, and \(n\) is the number of data points or observations made.

b. Range (dispersion) is the simplest statistic used to describe the spread of data about the mean. It is calculated by subtracting the smallest observation or value from the largest.

c. Standard deviation (SD) is the most commonly used statistic in the laboratory describing dispersion of groups of single observations. SD is the square root of

Box 1–2 Determining the Molarity of a Solution

PROBLEM:
What is the molarity of solution that contains 32 g of HCl in 300 mL of water?

ANSWER:
Final unit of measure needed: mol/L
Units of measure used: g/mL
Grams of HCl in 1 mole: 36.5

\[
\frac{32 \text{ g HCl}}{300 \text{ mL}} \times \frac{1000 \text{ mL}}{1 \text{ L}} \times \frac{1 \text{ mole}}{36.5 \text{ g HCl}} = 2.9 \text{ mol/L}
\]
**Box 1–3 Calculating Molecular Weight for Hydration**

**PROBLEM:**
How much CuSO₄·5H₂O must be weighed in order to make 1 L of 0.5 M CuSO₄?

**ANSWER:**
CuSO₄·H₂O has five water molecules, which add 90 g to the original 160 g/mole. Therefore, CuSO₄·5H₂O has a gmw = 250 g.

\[
\frac{250 \text{g CuSO}_4 \cdot 5\text{H}_2\text{O}}{\text{mol}} \times \frac{0.5 \text{ mol}}{1 \text{ L}} = 125 \text{ g/L}
\]

The variance. It is calculated by adding the squares of the differences between the individual results and the mean, dividing by n-1, and calculating the square root.

d. The coefficient of variation (CV) is a comparison of the relative variability in two sets of values, because not all laboratory data are expressed in similar units of measure or concentrations. It is expressed as a percentage and is calculated as follows:

\[
CV\% = \frac{SD}{\text{mean}} \times 100\%
\]

or

\[
\frac{SD}{\text{mean}} (100\%)
\]

**C. Laboratory automation and computer systems**

1. Automation in the clinical chemistry laboratory context is the mechanization of chemical analysis to minimize manual manipulation. For example, one chemistry analyzer uses a dry slide technology for sample handling and measurement, whereas another uses a closed-system cuvette for holding and mixing sample and reagent.

   a. The advantages and disadvantages associated with automation are shown in Table 1–1.

2. There are two basic approaches to automation in use today.
   a. Continuous flow analyzers use liquid reagents pumped through a continuous system of tubing. Each sample is introduced in a sequential manner.
   b. Discrete analyzers house samples and reagents in separate containers. Multiple tests can be performed on a single sample (random access analysis), or one test can be selected to perform on multiple samples (batch analysis).

3. Laboratory information system (LIS) is a system of computer software designed to handle laboratory data.
   a. The functions of an LIS include:
      (1) Database of patient information
      (2) Compilation of specimen test results
      (3) Production of patient reports

\[\text{Figure 1–1 Gaussian or normal distribution, SD = standard deviation.}\]
Table 1–1 Advantages and Disadvantages of Automation

<table>
<thead>
<tr>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Increased work capacity per unit of time</td>
<td>Initial costs</td>
</tr>
<tr>
<td>Minimized variability</td>
<td>Discontinuity of product</td>
</tr>
<tr>
<td>Reduced errors caused by manual manipulations</td>
<td>Technical skill required</td>
</tr>
<tr>
<td>Reduced sample volumes</td>
<td></td>
</tr>
<tr>
<td>Reduced consumable costs</td>
<td></td>
</tr>
</tbody>
</table>

(4) Production of ancillary reports
(5) Data storage

b. An LIS achieves its function via a central computer, a number of input/output devices, and the computer software.

D. Spectrophotometry and light emission techniques

1. A spectrophotometer is an instrument that measures the transmitted light of a solution and allows the operator to read the absorbance of the solution on a meter. The components of a spectrophotometer include the following:

   a. The light source provides radiant energy.
      (1) Tungsten lamps are the typical source in most spectrophotometers.
      (2) Deuterium (and hydrogen) lamps are used in spectrophotometers that examine the ultraviolet (UV) spectrum.
      (3) Mercury lamps are used in high-performance liquid chromatography spectrophotometers.

   b. The monochromator isolates the wavelength of interest. Examples include:
      (1) Colored glass filters
      (2) Interference filters
      (3) Prisms
      (4) Diffraction gratings

   c. The sample cell contains the solution in:
      (1) Cuvettes
      (2) Tubing (typical in automated equipment)
      (3) Plastic packs

   d. The photodetector converts radiant energy to electrical energy. Three types of photodetectors are:
      (1) Photocell (barrier-layer cell)
      (2) Phototube
      (3) Photomultiplier tube

2. Background

   a. Photons. Electromagnetic radiant energy is described in terms of wavelike properties, specifically as photons, which are discrete packets of energy traveling in waves.

   b. Wavelengths. A wavelength ($\lambda$) of electromagnetic energy is the linear distance between successive wave peaks and is usually measured in units of nanometers ($10^{-9}$ m).
      (1) Frequency is the number of wave peaks per given unit of time.

   c. The electromagnetic spectrum has a large range of wavelengths. Gamma rays and x-rays have very long wavelengths, whereas UV rays inhabit the portion of the electromagnetic spectrum from 10 to 400 nm. The visible spectrum lies between 400 and 800 nm. Violet light has the longest wavelength of the visible spectrum, followed by blue, green, yellow, orange, and red (VIBGYOR, ROY G. BIV). The infrared spectrum lies above 800 nm, and the shortest wavelengths are microwaves.

   d. Excitation. Interactions of light with matter occur when a photon intercepts an atom, ion, or molecule. The photon is absorbed, and the energy of the photon changes the matter (excitation). Some compounds are able to dissipate the absorbed energy, causing changes in the electronic state of the molecule, which may be reversible or irreversible.
energy as radiant energy upon return to a nonexcited state. **Excitation** can involve any of the following:

1. Movement of an electron to a higher energy state
2. Change in covalent bond vibrations
3. Change in covalent bond rotations

**Beer’s law** states that the concentration of a substance is directly proportional to the amount of radiant energy absorbed:

\[ A = abc \text{ or } ebc \]

where \( a \) (or \( e \)) is molar absorptivity (a constant for a given molecule); \( b \) is the length of the path traveled by the light; and \( c \) is the concentration of absorbing molecules.

**Standard curve.** In clinical chemistry, concentrations of unknown solutions are determined by plotting the absorbance of standard solutions (concentrations known) versus the concentrations of the standard solution, which creates a standard curve.

3. **Types of spectrophotometry**

   a. **Absorption spectrophotometry** is defined as the measurement of radiant energy absorbed by a solution. This measurement can be related to the concentration of a substance in the solution.

   1. Every solution has an ability to absorb and transmit light, and only transmitted light can be measured. **Transmittance** is defined as the proportion of incident light that is transmitted and is usually expressed as a percentage:

   \[ \%T = \frac{I}{I_0} \times 100 \]

   where \( I \) is the transmitted radiant energy, and \( I_0 \) is the original incident radiation. Transmittance varies inversely and logarithmically with the concentration of the solution.

   2. **Absorbance** is calculated as follows:

   \[ A = 2 - \log \% T \]

   The absorbance is the critical measure used in the calculation of concentration (Beer’s law).

   b. **Atomic absorption spectrophotometry (AAS)** measures concentration through the detection of absorbance of electromagnetic radiation by atoms instead of molecules. It is used to measure concentration of metals that are not easily excited.

   1. **Principle.** An element of interest is dissociated from its chemical bonds in the flame; then it is in an unexcited state. At this low energy, the atom can absorb radiation at a narrow specific bandwidth. A wavelength of light (emitted by a light source) specific for the atom is absorbed by the low-energy atoms in the flame, resulting in a decrease in the intensity of the light measured by the detector.

   2. **Components**

      a. The light source (hollow cathode lamp)
      b. Flame (produced by a burner head)
      c. Monochromator
      d. Photodetector (photomultiplier tube)

   c. **Nephelometry** is a method of measuring concentration in terms of light energy scattered in a forward direction by small particles in solution. The intensity of the scattered light is directly proportional to the number of particles in solution.

   d. **Turbidimetry** is a photometric measurement of unscattered light passing through a colloidal solution of small particles. It is essentially a measurement of blocked light, and the amount of blocked light is directly proportional to the number of particles in solution.

   e. **Fluorometry** is the photometric measurement of light emitted by a substance that has been previously excited by a source of UV light. After it is excited and driven into a higher energy state, a molecule loses energy by fluorescing. The amount of light emitted is proportional to the concentration of the substance in solution.
E. Electrochemistry and osmometry

1. Electrochemistry is the measurement of electrical signals associated with chemical systems that are incorporated into an electrochemical cell (i.e., electrodes and solution in which they are immersed).
   a. In an anode/cathode system, electrons spontaneously flow from an electrode of high electron affinity to an electrode of low electron affinity, if the electrodes are connected via a salt bridge.
   b. Each electrode is characterized by a half-cell reaction and a half-cell potential (voltage). The electrode from which electrons flow is called the anode. The electrode accepting the electrons is the cathode.

2. Potentiometric methods. The measurement of voltage potentials is based on the measurement of a potential (voltage) difference between two electrodes immersed in solution under the condition of zero current electrochemical measurements. There are various systems used for measuring these potentials.
   a. A pH meter is a potentiometric apparatus used to measure the concentration of hydrogen ions in solution. It measures the potential difference between one half-cell and a reference electrode.
      (1) One of the electrodes (one half-cell), the indicator electrode, is sensitive to and responds to changes in concentration of a particular ion species in the solution in which the electrode is immersed.
      (2) A second electrode (another half-cell), the reference electrode, has a potential that does not change (i.e., is not influenced by the activity of the ion being measured). It is an electrochemical half-cell that is used as a fixed reference for the measurement of cell potentials. Examples include:
         (a) Standard hydrogen electrode
         (b) Saturated calomel electrode
         (c) Silver/silver chloride
      (3) The indicator electrode is in an electrochemical half-cell that interacts with the analyte of interest. Examples include:
         (a) Ion-selective electrodes (ISE) measure a potential across a membrane specific for a certain analyte.
         (b) Glass-membrane electrodes are a type of ISE most commonly used for pH measurement.
   b. Coulometry is the measurement of the amount of electricity passing between two electrodes in an electrochemical cell. The amount of electricity is proportional to the amount of a substance produced or consumed by oxidation/reduction at the electrodes.
   c. Amperometry is the measurement of the current flowing through an electrochemical cell when a potential is applied to the electrodes.

3. Osmometry is the measurement of particle concentration that is related to the osmotic pressure of the solution. Osmotic pressure regulates the movement of a solvent across a membrane.

4. Osmolality describes the number of moles of particle per kilogram of water and depends only on the number of particles, not on what types of particles are present.
   a. The colligative properties of a solution are related to the number of solute particles per kilogram of solvent. Colligative properties include:
      (1) Osmotic pressure
      (2) Boiling point
      (3) Vapor pressure
      (4) Freezing point
   b. Colligative properties change as the number of particles in the solution change. In the clinical chemistry laboratory, vapor pressure and freezing point are the colligative properties of interest. These can be measured in an osmometer.
      (1) Freezing point depression. The more particles in solution, the lower the freezing point of the solution.
      (2) Vapor pressure depression. Increased particles in a solution prevent solvent evaporation.
c. Osmolal gap is the difference between the calculated osmolality and the actual measured osmolality.

1. The formula for calculated plasma osmolality is:

\[ 2 \times \text{Na (mEq/L)} + \frac{\text{Glucose (mg/dL)}}{18} + \frac{\text{BUN (mg/dL)}}{2.8} = \text{mOsm/kg} \]

2. If the osmolal gap is >0, there is an indication of an abnormal concentration of unmeasured substances (typically, ethanol) in the blood.

F. Pre-analytical variables in laboratory testing affect the outcome of specimen analysis and includes any event that affects specimen integrity, its collection, transport, or handling prior to analysis. Within a laboratory and phlebotomy area, approved procedure manuals that address patient identification (usually two types are required) and collection of each type of specimen that is tested by that laboratory must be available.

1. Specimen collection. Inaccurate specimen type and mislabeled specimens are the most common pre-analytical variables encountered in the laboratory.
   a. Evacuated blood tubes. Order of the draw is critical to avoid cross-contamination with anticoagulants (typically steroid tubes, then sodium citrate tubes followed by serum collection tubes, then heparin tubes, EDTA and glycolysis-inhibiting tubes are collected in that order). Samples collected for blood gas analysis have very specific requirements (see Section IV, C, 5f below).
   b. Urine specimens have specific collection requirements (see Chapter 9) as do specimens for bacteriological studies (see Chapter 7). Other specimen types require unique collection, transport, and storage.

2. Specimen transport is important in cases when samples must remain cold or on ice, such as samples required for blood gas analysis. Sample storage and preservation prior to analysis is also an important pre-analytical variable, particularly for urine specimens or samples that must be stored long term before testing.

G. Post-analytical interpretation is an essential component of quality analytical outcome. Use of appropriate control samples are the first step to quality postanalytical interpretation (see Chapter 11). Interpretation of laboratory results is typically the role of the physician; however, the quality of results that the physician sees remains the responsibility of the laboratory.

II. SPECIAL METHODS IN CLINICAL CHEMISTRY

A. Electrophoresis is the migration of charged particles in some medium (either liquid or solid) when an electrical field is applied. Depending on the charge of the molecules, negatively charged particles migrate toward the positive electrode (anode), and positively charged particles migrate toward the negative electrode (cathode).

1. Migration rate depends on:
   a. Charge of the molecule, which is directly proportional to rate of movement
   b. Size of the molecule, which is inversely proportional to rate of movement
   c. Electrical field, in which increased current increases migration rate
   d. Ionic strength of buffer, in which increased ionic strength decreases migration rate
   e. pH of buffer, in which decreased pH slows migration
   f. Viscosity of supporting medium, which is inversely proportional to migration rate
   g. System temperature, in which high temperature can denature protein and slow migration

2. Analytic electrophoretic procedures include protein electrophoresis and isoenzyme electrophoresis.
   a. Protein electrophoresis
      1. The principle of protein electrophoresis
A Concise Review of Clinical Laboratory Science

(a) Proteins are amphoteric (i.e., they can have positive or negative charge because of their acidic and basic side chains).
(b) The isoelectric point of protein is the pH at which a protein has no net charge.
(c) At pH 8.6, proteins are negatively charged and migrate toward the anode.
(d) If the buffer pH is higher than the isoelectric point of protein, the protein carries a negative charge and migrates toward the anode.

2. The methodology of electrophoresis

(a) A support medium (agarose gel or cellulose acetate) is put in contact with the buffer.
(b) A sample is applied to the medium.
(c) A constant current or voltage is applied, and particles are allowed to migrate and separate.
(d) The support is fixed and stained to visualize protein bands.

b. Isoenzyme electrophoresis is typically performed to visualize the isoenzymes of some clinically relevant enzymes.

1. The principle of isoenzyme electrophoresis is similar to that of protein electrophoresis because isoenzymes are proteins. The procedure is performed at a pH of 8.6, and the most negatively charged particles migrate toward the anode.
2. The methodology involved in isoenzyme electrophoresis is similar to that used for protein electrophoresis.

B. Immunoassay is a chemical assay based on the highly specific and tight, noncovalent binding of antibodies to target molecules (antigens). Immunoassay is typically useful when the endogenous concentration of an analyte is very low.

1. Components in the immunoassay system include antigens and antibodies.
   a. An antigen (ag) is a substance that can elicit an immune response (production of a specific antibody) when injected into an animal. The antigen is typically the analyte of interest.
   b. An antibody (ab) is an immunoglobulin formed in response to a foreign substance (antigen). The antibody is the most important component of this system, because it determines the sensitivity (ability to detect small amounts) and specificity (the degree of uniqueness of the ag-ab reaction) of the procedure.

2. Immunochemical labels are necessary to detect the ag-ab reaction.
   a. Enzyme labels are attached to the antibody. With the addition of a Chromagen, they allow the immunoassay results to be quantitated colorimetrically.
   b. Fluorescent labels are attached to the antibody and are detected when a photon is released from a fluorescent molecule that is excited from its ground state to a higher state and then returns to the ground state. A drawback of this system lies with the autofluorescence of serum.
   c. Chemiluminescent labels are compounds that undergo a chemical reaction and form an unstable derivative. Upon return to the ground state, they release energy in the form of visible light. The light is measured by a luminometer, and light intensity is related directly to the concentration of the reactants.
   d. Radioisotope labels are compounds that have the same atomic number but different weights than the parent nuclide (e.g., $^{125}$I, $^{14}$C). Radioisotopes decay to form a more stable isotope. In the process, they emit energy in the form of radiation (electromagnetic gamma rays) that can be detected and quantitated.

3. Immunoassay methodologies are based on the label attached to the antigen or antibody (Table 1–2).

C. Chromatography is a technique used to separate complex mixtures on the basis of different physical interactions between the individual compounds and the stationary phase of the system (a solid or a liquid - coated solid). The goal of this technique is to produce “fractions” for quantification.

1. Mechanisms of separation are based on the interactions of solutes with mobile and stationary phases.
### Table 1–2: Methods of Immunoassay

<table>
<thead>
<tr>
<th>Method</th>
<th>Basis</th>
<th>What is Labeled</th>
<th>Use</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme-linked immunosorbent assay (ELISA)</td>
<td>Enzyme-based</td>
<td>Antigen in some methods; antibody in others</td>
<td>Hormone testing</td>
</tr>
<tr>
<td>Enzyme-multiplied immunoassay technique (EIA, EMIT)</td>
<td>Enzyme-based</td>
<td>Antigen</td>
<td>Drug monitoring</td>
</tr>
<tr>
<td>Fluorescence-polarized immunoassay (FPIA)</td>
<td>Fluorescence-based</td>
<td>Antigen</td>
<td>Hormone testing</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Surfactant/albumin ratio</td>
</tr>
<tr>
<td>Fluorescent immunoassay (FIA)</td>
<td>Fluorescence-based</td>
<td>Antigen (fluorescence is proportional to concentration of analyte)</td>
<td>Catecholamine testing</td>
</tr>
<tr>
<td>Radioimmunoassay (RIA)</td>
<td>Radiation-based</td>
<td>Antigen</td>
<td>Hormone testing; drug monitoring</td>
</tr>
<tr>
<td>Immunoradiometric assay (IRMA)</td>
<td>Radiation-based</td>
<td>Antibody</td>
<td>Hormone testing; drug monitoring</td>
</tr>
</tbody>
</table>

### Adsorption Chromatography (liquid-solid chromatography)

Adsonption chromatography is based on the competition between the sample and the mobile phase for binding sites on the solid (stationary) phase. Molecules that are most soluble in the mobile phase move fastest.

### Partition Chromatography (liquid-liquid)

Partition chromatography depends on the solubility of the solute in nonpolar (organic) or polar (aqueous) solvents.

### Ion-exchange Chromatography

Ion-exchange chromatography involves the separation of solutes by their size and the charge of the ionic species present. The stationary phase is a resin (can be cationic with free hydrogen ions or anionic with free hydroxyl ions present). Anion- and cation-exchange resins mixed together are used to deionize water.

#### Chromatographic Procedures

- **Thin-layer Chromatography (TLC)**
  - A layer of absorbent material is coated on a plate of glass, and spots of samples are applied. The solvent is placed in a container and migrates up the thin layer by capillary action. Separation is achieved by any of the previously discussed modes (see above). Sample movement is compared with the standard, and fractions are calculated using retention factor ($R_f$), which is unique for specific compounds:

  \[
  R_f = \frac{\text{distance component moves}}{\text{total distance}} - \frac{\text{distance solvent front moves}}{\text{total distance}}
  \]

- **High-performance liquid chromatography (HPLC)**
  - HPLC provides quantitative results. It is highly sensitive and specific. Apparatus consists of a pressure pump; a gel-filled column; a sample injector; a detector that monitors each component (e.g., spectrophotometers, amperometric detectors); and a recorder. Sample and solvent are pushed through the column, and the resulting eluent is read by the detector. The peaks that are detected and printed are specific and distinctive for each compound that is analyzed by HPLC.

- **Gas Chromatography (GC)**
  - Gas chromatography separates mixtures of volatile compounds. It can have a solid or liquid stationary phase. The setup is very similar to HPLC, except the solvent is a gas, the sample is vaporized, and detectors are thermal conductivity or flame ionization. A special detector can be a mass spectrometer (MS), which measures the fragmentation patterns of ions (GCMS) and is used in drug identification. Gas chromatography is divided into two categories:

  1. **Gas-solid chromatography**, in which the absorbent is a solid material;
  2. **Gas-liquid chromatography**, in which the absorbing material is a liquid coated on a solid phase.
III. BASIC ANATOMY AND PHYSIOLOGY

A. Kidney

1. Renal structure can be viewed both macroscopically and microscopically.
   a. The macroscopic structure of the kidney consists of the cortex, medulla, and pelvis.
   b. The microscopic structure of the kidney includes the nephron, which is considered to be the functional unit of the kidney, and consists of the:
      (1) Glomerulus (made of arterioles surrounded by the distended end of a renal tubule in the renal cortex)
      (2) Proximal tubules (located in the cortex)
      (3) Henle’s loop (descending and ascending limbs in the renal medulla)
      (4) Distal tubules (in the cortex)
      (5) Collecting tubules (collect urine from distal tubules to drain into the renal pelvis)

2. Renal physiology is based on the function of each microscopic component.
   a. Glomerular function is to strain proteins from the plasma and produce a “protein-free” filtrate that becomes urine.
      (1) The glomerular filtration rate (GFR) equals 125 to 130 mL protein-free fluid formed per minute.
      (2) Clearance indicates the number of milliliters of plasma from which the kidney can remove all of a given substance in 1 minute. A request for “clearance” is a request for assessment of glomerular filtration rate.
      (3) Plasma renal flow is the number of milliliters of plasma passing through the kidney in 1 minute; normal is 625 mL/min.
   b. Tubular function is to resorb certain substances back into the body. The proximal tubule resorbs 75% of water, sodium, much of glucose, amino acids, certain ions, and small molecules. Some substances have a maximum concentration in plasma, so the tubule cannot resorb it all. Excess substance spills over into urine (e.g., glucose). The proximal tubule allows for the elimination of urea and creatinine.
   c. The Loop of Henle adjusts urine osmolality to keep the urine watery.
   d. The distal tubule resorbs some salt, water, and bicarbonate, but eliminates uric acid, ammonia, and hydrogen ions. The distal tubule is under hormonal control.
   e. The collecting ducts are under hormonal control for resorption of water and sodium.

3. The renal system functions to maintain a balance of water, ions, and pH; to eliminate nonprotein nitrogens; and to synthesize certain hormones.
   a. Water balance is maintained by ingestion of water (controlled by the brain thirst center) and excretion/resorption of water in the renal tubules under hormonal control by antidiuretic hormone (ADH).
   b. Ionic balance of sodium, potassium, phosphate, calcium, and magnesium is maintained by tubule resorption under hormonal control (aldosterone). Chloride is passively resorbed with sodium.
   c. Acid-base balance is controlled by kidney conservation of bicarbonate ions and removal of metabolic acids (H+) to conserve blood pH level.
   d. Nonprotein nitrogen (e.g., urea, creatinine, uric acid) is eliminated or filtered by the glomerulus. Some urea and uric acid is reabsorbed into the blood.
   e. The kidneys synthesize three hormones and one enzyme. Kidneys also serve as a site for the hormonal action of aldosterone and ADH.
      (1) Renin is a vasoconstrictor synthesized in the renal medulla.
      (2) Prostaglandins are synthesized in the kidney and affect renal blood flow.
      (3) Erythropoietin increases heme production and iron insertion into red blood cells (RBCs) and is formed in conjunction with an enzyme made in the kidney.
      (4) Dihydroxycholecalciferol hydrolase activates Vitamin D into a usable form.
4. Renal system disorders affect the glomerulus, the tubules, or other components of the system (for more details, see Chapter 9).
   a. Glomerular diseases affect portions of the glomerular structure.
      (1) Glomerulonephritis is related to group A β-hemolytic streptococcal infections. Immune complexes damage the structure of the glomerulus, leading to anemia, uremia, and edema.
      (2) Nephrotic syndrome refers to the increased permeability of the glomerular cell basement membrane, which leads to proteinuria and edema.
   b. Tubular diseases occur in all renal diseases as GFR falls and affect acid-base balance.
   c. Urinary tract infections are bacterial infections that produce bacteriuria and pyuria.
   d. Renal calculi (kidney stones) are deposits of calcium and uric acid that follow urinary tract infections and lead to hematuria.
   e. Renal failure can be acute or chronic and affects many chemistry analytes.
      (1) Acute renal failure is typically caused by cardiovascular system failure (prerenal), necrosis of the tubular system (renal), or obstruction of the lower urinary tract (postrenal). This condition leads to oliguria, proteinuria, and hematuria.
      (2) Chronic renal failure results from the chronic loss of excretory and regulatory functions. Causes vary from chronic glomerulonephritis to obstructive uropathy to renal vascular disease.

B. Liver

1. Hepatic structure can be viewed both macroscopically and microscopically.
   a. The macroscopic view of the liver reveals a bilobed organ richly vascularized with two main supply vessels: the hepatic artery and the portal vein.
   b. The microscopic structural and functional unit of the liver is the lobule, which consists of:
      (1) Cords, or hepatocytes, that surround a central vein
      (2) Sinusoids consisting of blood spaces lined with endothelial cells and Kupffer’s cells that surround the cords, which drain into a central vein
      (3) Bile canaliculi, or small channels between hepatocytes that carry bile formed by the hepatocytes to the bile ducts

2. Hepatic physiology depends on the components of the liver.
   a. The excretory/secretory function serves to process substances that have been absorbed from the gut and then transferred to the blood for use by other cells of the body.
      (1) Bile is involved with processing of lipids. It is composed of bile acids, salts, pigments, and cholesterol. Bile salts are formed in the hepatocytes, excreted into the bile canaliculi, and stored in the gall-bladder. Eventually, they are dumped into the duodenum to aid in the digestion of fats. Bile salts are then reabsorbed and re-excreted.
      (2) Bilirubin is the major bile pigment formed from the breakdown of hemoglobin when aged RBCs are phagocytized. The following steps occur: Hemoglobin is broken down into globin (reused) + iron (reused) + porphyrin (excreted) + biliverdin (reduced to bilirubin).
         (a) In the liver, bilirubin is conjugated (esterified) and becomes water soluble. This substance floats out of the bile canaliculi and into the gut, where it is eventually broken down to form urobilinogen, which is oxidized to produce urobilin and excreted in the stool.
         (b) Some urobilinogen is excreted by the kidney. There is some unconjugated bilirubin in the serum; increased bilirubin in the blood produces jaundice.
   b. Synthetic function. Albumin, α- and β-globins, blood-clotting factors, glycogen, carbohydrates, fat, some lipids, ketones, and some enzymes are synthesized in the hepatocytes.
   c. Detoxification function. Hepatocytes have the capability to conjugate (and thus inactivate) a substance or to modify it chemically.
d. **Storage function.** Iron, glycogen, amino acids, and some lipids are stored in hepatocytes.

3. **Hepatic disorders**
   a. **Jaundice,** which causes yellowish discoloration of skin, is caused by abnormal bilirubin metabolism or by retention of bilirubin.
      1. **Prehepatic jaundice** is the result of excessive bilirubin presented to the liver. It can occur in newborns and in people with hemolytic anemia or ineffective erythropoiesis. This condition produces increased serum unconjugated bilirubin.
      2. **Hepatic jaundice** is present in people with hepatobiliary disease. This disorder exhibits increases in both unconjugated and conjugated bilirubin levels.
      3. **Posthepatic jaundice** is produced by obstruction of the flow of bile into the gut either by gallstones or a tumor, which causes increased conjugated bilirubin levels in serum and urine, but low urobilinogen levels in urine and colorless stool.
   b. **Cirrhosis** is defined as destruction of the liver’s architecture. The leading cause of this condition is alcohol abuse.
   c. **Reye’s syndrome** is liver destruction caused by viral infection, although the etiology of this disease is unknown. Ammonia accumulates in the liver and blood.
   d. **Hepatitis** is defined as inflammation of the liver and subsequent hepatocellular damage caused by bacterial infection, drugs, toxins, or viral infections. **Types of viral hepatitis** include:
      1. **Hepatitis A** ("infectious" hepatitis), also known as hepatitis A virus (HAV), is transmitted by contamination of food and water.
      2. **Hepatitis B** ("serum" hepatitis), or hepatitis B virus (HBV), has an outer coat called the HBV surface antigen (HBsAg) that covers the HBV core antigen (HBcAg). Hepatitis B is transmitted through parenteral injection or through exchange of bodily secretions, as occurs during sexual intercourse.
      3. **Hepatitis C** (HCV) is a non-A, non-B hepatitis that is transmitted parenterally through blood transfusions, body piercings, and inoculations and has become more common. It is the leading cause of chronic liver disease.
      4. Delta hepatitis can cause infection only in patients infected with hepatitis B.

C. **Gastrointestinal (GI) tract and pancreas.** Anatomically, the GI tract is composed of five regions: the mouth, stomach, duodenum, jejunum-ileum, and large intestine.

1. Gastric and GI functions are important to consider in the diagnosis of digestive disorders.
   a. **Digestion** is the chemical processing of food into an absorbable substance. It begins in the mouth and continues in the stomach and duodenum.
      1. **Gastric fluid** in the stomach is composed of hydrochloric acid, pepsin, intrinsic factor, and mucus. The pH of this fluid is <3. The secretion of gastrin by gastric cells stimulates gastric fluid secretion.
      2. **Intrinsic factor,** produced in the parietal cells of the stomach, is required for the transport of vitamin B12 across the intestinal wall.
   b. **Absorption** is the process that allows digested food to enter the body. This process occurs in the small intestine.

2. **GI function tests** evaluate the level of function and determine the primary cause of malabsorption syndrome.
   a. **Gastric fluid analysis** serves to:
      1. Determine pH of gastric fluid, with low pH (achlorhydria) indicative of pernicious anemia
      2. Detect hypersecretion of gastric fluid caused by a secreting tumor (e.g., Zollinger-Ellison syndrome)
      3. Check acid secretion in treatment of ulcers
      4. Verify vagotomy (i.e., severing nerves to stomach for treatment of ulcers)
   b. **Lactose intolerance test** examines whether lactose is formed normally in gastric cells. The procedure involves ingestion of a lactose cocktail followed by glucose analysis. Little or no increase in serum glucose indicates lactase deficiency.
D. The pancreas is a highly vascularized organ connected to the small intestine by the ampulla of Vater. It is considered both an endocrine gland that synthesizes hormones and an exocrine gland that provides digestive enzymes to aid in digestion.

1. Pancreatic functions
   a. Endocrine function is performed in the islets of Langerhans. These cell groups are composed of three types of cells.
      (1) **α** cells produce glucagon, which stimulates the conversion of glycogen into glucose (glycogenolysis).
      (2) **β** cells are responsible for making insulin, which functions to promote glycogenesis and thereby lowers glucose levels.
      (3) **δ** cells produce gastrin and somatostatin.
   b. Exocrine function is performed by the acinar cells. These cells produce the following enzymes:
      (1) Amylase, which breaks down starch and glycogen and is used to diagnose acute pancreatitis;
      (2) Lipase, which hydrolyzes fats to produce alcohols and fatty acids with elevated levels present in people who have acute pancreatitis; and
      (3) Trypsin, which is a proteolytic enzyme (functions in protein breakdown).

2. Pancreatic disorders typically result in decreased secretion of enzymes or hormones.
   a. Cystic fibrosis is an autosomal recessive genetic disorder characterized by pulmonary disease and intestinal malabsorption caused by lack of pancreatic enzyme secretion.
   b. Pancreatitis (inflammation of the pancreas) is associated with alcohol abuse or gallbladder disease and also occurs in patients with lipid disorders and is caused by the release of pancreatic enzymes from cells into the surrounding pancreatic tissue.
   c. Diabetes mellitus is a multifactorial disease that occurs when the pancreas can no longer produce insulin, which leads to hyperglycemia. This disorder almost always destroys the **β** cells in the islets. In type II diabetes mellitus, cells no longer are sensitive to insulin and glucose remains in the blood.
   d. Pancreatic cancer is a fatal disease that affects the ducts in the pancreas. Insulinoma is a tumor of the **β** cells in the islets that leads to increased circulating insulin and hypoglycemia.

3. Tests of exocrine pancreatic function
   a. Secretin test determines the secretory capacity of the pancreas. It involves intubation and gathering of pancreatic fluid after stimulation with secretin, followed by measurement of fluid volume.
   b. Quantitative fecal fat examination determines the presence of increased fats in feces (steatorrhea), which is a disorder almost always associated with exocrine pancreatic insufficiency. A 72-hour fecal specimen is collected, and the fats extracted with ether and weighed. A screening procedure involves mixing a small amount of fecal specimen with a fat-soluble stain and examining the specimen microscopically for lipid droplets.
   c. Sweat electrolytes are measured to diagnose cystic fibrosis. Pilocarpine nitrate is used to stimulate sweating on skin which is collected on a small disc. Sweat is eluted from the disc and analyzed for chloride and sodium content. Newborn screening programs and genetic tests that assess the presence of genetic alterations in a number of genes related to cystic fibrosis are also available (see Chapter 10).
   d. Enzyme testing for amylase and lipase is performed using a variety of methodologies. These are listed below.

E. The cardiovascular system is composed of the heart and blood vessels. Some include the pulmonary system because of the extensive connections between the heart and lungs.

1. The heart is a four-chambered muscular organ. Blood passes first through the right, or pulmonary, side of the heart to be oxygenated in the lungs and then is returned to the left, or systemic, side that boosts pressure for the circuit of blood around the body.
2. The microscopic anatomy of the heart includes the myocardium that is made up of cardiac muscle fibers.
   a. Myocardium is the muscular tissue of the heart. Myocardium is composed of cardiac muscle fibers interspersed with blood vessels, lymphatics, and nerves. The fuel of the heart muscle tissue is free fatty acid.
   b. Cardiac muscle is found only in the heart. Cardiac muscle fibers synthesize specific proteins (troponin for example) that can be assessed in blood following muscle cell injury. Myoglobin acts as the storage vessel for oxygen in muscle cells.

3. Cardiac dysfunction involves many parts of the heart and can begin in an area other than the heart itself. Heart failure takes many forms, such as congestive heart failure, coronary artery disease, and myocardial infarction (heart attack). Heart failure is based on the functional anatomy of the heart. Failing hearts do not pump enough blood to sustain the body with oxygen.

IV. ANALYTES AND PATHOPHYSIOLOGY

A. Amino acids are defined as organic compounds containing both an amino (NH₂) group and a carboxyl (COOH) group. α-Amino acids are present in proteins; they differ in their side chains, which give individual amino acids their special properties. Proteins are macromolecules composed of covalently linked polymers of amino acids linked by peptide bonds in a head-to-tail fashion (Figure 1–2). Proteins are composed of carbon, oxygen, hydrogen, nitrogen, and sulfur.
   1. Essential amino acids must be supplied by dietary intake. These include valine, leucine, isoleucine, methionine, tryptophan, phenylalanine, threonine, lysine, and histidine.
   2. Ketoacids are produced by removal of an amino group from an amino acid. Ketoacids can be either:
      a. Glycogenic to generate glucose precursors; or
      b. Ketogenic to generate ketone bodies
   3. Aminoacidopathies are disorders that involve faulty amino acid metabolism.
      a. Phenylketonuria (PKU) is an inherited disorder causing lack of phenylalanine hydroxylase and the inability to convert phenylalanine to tyrosine, which results in the formation of phenylpyruvate. PKU causes mental retardation in children.
      b. Maple syrup urine disease (MSUD) is a disorder of decarboxylation of the ketoacids of leucine, isoleucine, and valine, which results in accumulation of ketoacids in blood, urine, and spinal fluid. MSUD causes mental retardation or death in infants.
      c. Homocystinuria is caused by impaired enzyme activity, which results in elevated levels of homocysteine and methionine in plasma and urine.
   4. Protein structure. The principal plasma proteins include albumin and the globulins. Other protein fractions include fibrinogen and complement.
      a. Albumin is responsible for the osmotic pressure of plasma and serves as a transport protein. Prealbumin migrates ahead of albumin during electrophoresis and transports thyroid hormones.
      b. The globulins are insoluble in water. There are several globulin fractions, based on their electrophoretic mobility. These include:
         (1) α₁-Globulins (α₁-fetoprotein, α₁-antitrypsin)
         (2) α-Globulins (haptoglobin, ceruloplasmin, and α₂-macroglobulin)
         (3) β-Globulins (transferrin, C-reactive protein)
         (4) γ-Globulins [immunoglobulins (Ig) G,A,M,D,E]
5. Synthesis. Proteins are synthesized in the liver (serum proteins) or by B-cells (immunoglobulins). Protein formation is specified by the DNA in each type of cell (hepatic or plasma).

6. Protein catabolism takes place in the gastrointestinal tract, kidneys, and liver. Protein disintegrates into constituent amino acids, which are further deaminated into ketoacids and ammonia. Ammonia is used in the formation of urea.

7. Classification. Proteins are classified as simple or conjugated.
   a. Simple proteins are peptide chains that hydrolyze to amino acids.
   b. Conjugated proteins are composed of protein (apoprotein) and a nonprotein substance (referred to as prosthetic groups), such as lipid (forms lipoproteins), carbohydrate (forms glycoproteins), or metals (form metalloproteins).

8. Functions of protein include tissue nutrition, water distribution, plasma buffer, substance transport, and structural support.

9. Protein analysis determines either the total nitrogen content of the sample or the total protein. The normal reference range of serum protein is 6.5 to 8.3 g/dL.
   a. Total nitrogen determination analyzes both protein and nonprotein nitrogen.
   b. Kjeldahl’s method involves the conversion of protein nitrogen into ammonium ion. This classic method is not practical for routine use.
   c. Refractometry methods use the refractive index of a solution in the determination of solute concentration.
      (1) The principle states that the velocity of light changes when it passes between air and water, causing light to bend (refract). Therefore, the refractive index of water increases proportionally to the concentration of a solute in solution.
      (2) The solutes present in greatest concentration in serum are proteins. Therefore, this method provides an approximation useful as a rapid test, but error can occur in patients with hyperglycemia, lipemia, or azotemia.
   d. The biuret method is the most widely used method of protein determination. Analysis depends on the presence of peptide bonds.
      (1) The principle states that cupric (Cu²⁺) ions react with peptide bonds to form a violet color proportional to the number of peptide bonds present.
      (2) This method is widely used and easily automated.
   e. The dye-binding method is based on the ability of proteins to bind dyes. Albumin binds dye with the strongest affinity.
      (1) The principle of this method is that dye binds to protonated amine groups of amino acids, with absorption at 595 nm.
      (2) Dye-binding methods are used chiefly for albumin analysis. When albumin is positively charged, it binds to certain dyes, causing a shift in absorbance from free dye. Brom-cresol green is the most widely used dye, although brom-cresol purple is considered more specific in binding albumin.
   f. UV absorption is based on absorption of UV light by peptide bonds at 210 and 280 nm.
   g. Serum protein electrophoresis (SPE) relies on the separation of proteins based on their net electrical charges, size, properties of the support medium, and temperature of operation. This is a semi-quantitative method.
      (1) The principle of SPE states that when an electric field is applied to a medium containing charged particles, the negatively charged particles migrate toward the positive electrode (anode), and the positively charged particles migrate toward the cathode. At pH 8.6, most serum proteins have a negative charge. This separates the protein fractions in serum when an electrical field is applied.
      (2) On the support medium (cellulose acetate or agarose gel), the pattern of migration is as follows: Albumin is most anodic (because of its small size and large number of negative charges), then α₁-globulins, α₂-globulins, β-globulins, λ-globulins are most cathodic (Figure 1–3).
   h. Technical issues with electrophoresis include endosmosis, inappropriate buffer pH, unusual or atypical bands, distorted protein bands, and discontinuous zones.
Figure 1-3 Normal serum protein electrophoretic pattern.

(a) Electroendosmosis occurs when electrophoretic support media that are in contact with water take on a negative charge due to adsorption of hydroxyl ions. Macromolecules, such as immunoglobulins, in solution may remain immobile or even be swept back toward the opposite pole if they are insufficiently charged. Endosmosis is minimal in systems that use purified agarose, starch or polyacrylamide.

(b) Old buffer will exhibit pH changes caused by the electrolysis of water that accompanies electrophoresis. Changes in pH will cause proteins to lose amphotericity and migration will be affected.

(c) Atypical bands might be caused by artifact, such as hemolysis (heavy β-band) or fibrinogen (band close to application point) from a plasma sample. Denatured protein from a deteriorated serum will produce sharp irregular bands.

(d) Discontinuous zones or distorted bands can be caused by overapplication of sample, dirty applicators, wet gels, dirty electrodes, or dried out supports.

h. Capillary electrophoresis is a method in which the techniques of electrophoresis are carried out in a small-bore, fused silica capillary tube. This capillary tube serves as an electrophoresis chamber that is connected to a detector at its terminal end and to a power supply. Buffers include Tris, borate, acetate, formate, and phosphate. Capillary zone electrophoresis is the simplest form of electrophoresis and is able to resolve many analytes such as proteins, peptides, and amino acids.

i. Radial immunodiffusion (RID) is a quantitative method of immunoglobulin determination.
   (1) The principle of RID states that an agarose gel is saturated with a specific antibody solution. Antigen (serum) is applied and diffuses radially into the medium. At the point where antigen and antibody concentration are equal, a precipitin ring forms. Measurement of the diameter of the precipitin ring squared is compared with a standard curve of antigen concentration.
   (2) RID is also referred to as the Mancini technique or the Ouchterlony technique.

j. Nephelometry is used as a quantitative method for immunoglobulin analysis.
   (1) The principle states that immunoglobulins react with a specific antibody to produce an immunoprecipitate. Forward light scatter is measured.
   (2) Nephelometry is used for protein analysis of all body fluids.

k. Immunofixation electrophoresis (IFE) is a qualitative method for evaluation of immunoglobulins.
   (1) The principle of IFE states that proteins are electrophoresed into five zones as in SPE. Then, monospecific antiserum is added, and the support medium is stained for visual interpretation of bands.
   (2) IFE is used to analyze protein concentration in serum, urine, and other fluids.
I. Immunoelectrophoresis (IEP) is a qualitative method for the evaluation of immunoglobulins. Capillary electrophoresis can be used for IEP.

1. The principle of IEP states that proteins are electrophoresed into five zones similar to SPE. Then, antibody is added to produce precipitin arcs that are visually interpreted.

2. IEP is used to analyze serum, cerebrospinal fluid (CSF), and urine.

10. Other methods. Urinary proteins (for details, see Chapter 9) are analyzed by:

a. Qualitative dipstick tests for proteinuria, glycosuria, and other abnormal substances in the urine that rely on color change for interpretation.

b. Quantitative turbidimetric methods require that a protein precipitant is added to urine. The resulting turbidity is measured photometrically.

11. Protein disorders are the result of high or low serum protein levels or a dysfunction of the immunoglobulins.

a. Hypoproteinemia can be caused by kidney disease, blood loss, malnutrition, and liver disease.

b. Hyperproteinemia is observed in people with dehydration or excess production of γ-globulins.

c. Decreased serum albumin is caused by a variety of disorders, including poor diet, liver dysfunction, GI inflammation, and renal disease.

d. Specific globulin disorders include the following:

1. α1-Antitrypsin deficiency is caused by pulmonary disease, and increased levels are caused by inflammation or pregnancy.

2. Elevated levels of α1-fetoprotein during pregnancy may indicate neural tube defects, spina bifida in the fetus, or twins. Very high levels are found in individuals with liver cancer. Decreased levels during pregnancy indicate a risk of fetal Down’s syndrome.

3. Haptoglobin, an α2-macroglubulin, is increased in inflammatory conditions, burns, and rheumatic disease. Decreased levels are seen in people with transfusion reactions or hemolytic disease.

4. Ceruloplasmin, an α2-macroglobulin, is decreased in people with Wilson’s disease and states of malnutrition.

5. Transferrin levels are increased during iron deficiency anemia.

6. Immunoglobulin increases indicate infection, liver disease, Waldenström’s macroglobulinemia (IgM), multiple myeloma, or autoimmune reactions. These increases appear as a spike, either monoclonal or polyclonal, in the gamma region of SPE.

B. Water and electrolyte balance. Electrolytes are ions capable of carrying an electric charge. They can be either anions (negatively charged) or cations (positively charged). Total body water (TBW) makes up about 60% of male body weight and 50% of female weight. Two thirds of TBW is in the intracellular compartment and the other third is extracellular fluid (ECF); 25% of the extracellular fluid is plasma.

1. Sodium is the most abundant extracellular cation. It contributes to the osmolality of extracellular fluid and maintains the volume of ECF and cell size and shape. Sodium is essential for transmitting nerve impulses.

a. Regulation of sodium is performed by the renal system in two ways.

1. The renin-ADH system. Low blood volume (as in cardiac failure) induces secretion of renin, a vasoconstrictor, from the kidney, which raises blood pressure and causes production of ADH. In turn, fluid volume is increased by the retention of sodium.

2. The renin-aldosterone system. Low blood volume induces secretion of renin, which induces production of aldosterone by the adrenal glands. In turn, kidney reabsorption of sodium and retention of water increase.

b. Sodium disorders

1. Hyponatremia is low serum sodium caused by gastrointestinal loss, burns, or renal problems. Dilutional hyponatremia is a relative decrease of sodium caused by excess body water, as in nephrotic syndrome or cirrhosis.
2. Hypernatremia is increased sodium caused by excess water loss, as in sweating or diarrhea. Dehydration causes a relative increase in sodium.

2. Potassium is the major intracellular cation that regulates activity at the neuromuscular junction, as well as cardiac muscle contraction and pH.

   a. Regulation of potassium occurs in two ways:
      (1) Dietary intake, which controls the amount of potassium in the circulation
      (2) The renal system, which controls potassium by:
          (a) Aldosterone induces potassium reabsorption and secretion by the renal tubules by exchanging it for sodium.
          (b) A high concentration of hydrogen ions keeps potassium out of cells and induces its renal retention. Low hydrogen ion concentration allows more potassium ions to enter cells, which lowers serum potassium. Low hydrogen ion concentration also allows more potassium to be excreted by the kidney.
   b. Potassium disorders include:
      (1) Hypokalemia (low serum potassium), which is a result of decreased dietary intake, gastrointestinal loss, or renal dysfunction, can produce irregular heartbeat.
      (2) Hyperkalemia (high serum potassium) is rare. It occurs following excessive dietary intake, adrenal failure, blood transfusions, or crush injuries.

3. Chloride is the major extracellular anion that acts to maintain osmotic pressure, keeps the body hydrated, and maintains electric neutrality via interaction with sodium or carbon dioxide.

   a. Regulation of chloride depends on dietary intake, sodium concentration, and the chloride shift, which moves chloride into RBCs as bicarbonate diffuses out to produce electroneutrality.
   b. Chloride disorders include:
      (1) Hypochloremia (low serum chloride) is caused by salt loss during renal disease, diabetic ketoacidosis, or prolonged vomiting.
      (2) Hyperchloremia (elevated serum chloride) is caused by dehydration, acute renal failure, prolonged diarrhea with loss of sodium bicarbonate, and salicylate intoxication.

4. Bicarbonate is the second most abundant anion in the extracellular fluid. It is a major component of the blood buffering system, accounts for 90% of total blood carbon dioxide, and maintains charge neutrality in the cell.

   a. Regulation of bicarbonate is achieved by the kidneys, which are responsible for reabsorbing all bicarbonate as carbon dioxide.
   b. Bicarbonate (or total carbon dioxide) disorders include decreased levels observed during metabolic acidosis, renal failure, or diarrhea, and elevated levels due to carbon dioxide retention, as is observed during respiratory acidosis.

5. Primary methods of electrolyte determination include:

   a. Ion-selective electrodes that use a semipermeable membrane to develop a potential between two different ion concentrations
   b. Amperometric-coulometric titration for chloride determination, in which silver ions are combined with chloride; when excess free silver ions are noted, elapsed time is relative to the chloride concentration
   c. Atomic absorption spectrophotometry

6. Anion gap is the difference between unmeasured anions and unmeasured cations. The normal reference range for the anion gap is 6 to 18 mmol or mEq/L. It is useful in determining increases in unmeasured anions and is calculated as follows:

   \[
   \text{Anion gap} = (\text{Na}^+ + \text{K}^+) - (\text{Cl}^- + \text{HCO}_3^-)
   \]

C. Acid-base balance assures the maintenance of a constant blood pH (7.4) through physiologic buffers, the respiratory system, and the renal system.
1. Buffer systems protect the body against changes in hydrogen ion concentration. Three physiologic buffers act to maintain a constant pH:
   a. The carbonic acid-bicarbonate system
   b. Hemoglobin
   c. The phosphoric acid-phosphate system

2. The respiratory system acts to maintain acid-base balance. Oxygen is removed from oxyhemoglobin in the tissues. This allows for the acceptance of hydrogen ions, forming carboxyhemoglobin. In the lungs, carboxyhemoglobin recombines with bicarbonate to form carbonic acid, which breaks down to form carbon dioxide and water. The carbon dioxide is then expired by respiration. Thus, ventilation affects the pH of the blood; this system is called the “respiratory” component of acid-base balance.

3. The renal system controls bicarbonate concentration. The overall reaction results in the reabsorption of sodium and bicarbonate in the kidney tubules. These substances pick up excess hydrogen ions. This system is called the “nonrespiratory” or “metabolic” component of acid-base balance.

4. Acid-base disorders are considered in terms of the Henderson-Hasselbalch equation, which states acid-base relationships:

   \[ \text{pH} = \text{pK} + \log \left( \frac{c_A}{c_{HA}} \right) \]

   where A is the proton acceptor (base) and HA is the proton donor (acid).

5. Blood gas analyzers determine acid-base balance through the measurement of partial pressure of oxygen, carbon dioxide, and pH. Analyzers use electrodes as sensing devices, and bicarbonate and other parameters are calculated from the previously mentioned measurements using the Henderson-Hasselbalch equation. Oxygen saturation is calculated as well. Analyzers typically measure arterial blood gases (ABG).
   a. Specimen requirements for blood gas analysis include the following.
      1. Arterial blood is collected in a glass or plastic syringe. Capillary specimens can also be used (blood must be “arterialized”).
      2. Lyophilized or liquid heparin is the preferred anticoagulant.
      3. No air bubbles should exist in the sample because they lower the pCO2 value.
      4. The specimen must be placed on ice and transported to the laboratory in 15 minutes at 4°C and tested immediately. Otherwise, pH values decrease, and pCO2 values increase.
      5. Blood clots are unacceptable.

6. Acid-base disorders are classified according to their cause. Compensation occurs when pH becomes abnormal.
   a. Respiratory acidosis results from hypventilation, which causes a decrease in carbon dioxide elimination. Compensation occurs by the kidneys increasing the resorption of bicarbonate.
   b. Respiratory alkalosis results from an increase in ventilation, resulting in excessive elimination of carbon dioxide. Compensation occurs by the kidneys excreting more bicarbonate.
   c. Nonrespiratory (metabolic) acidosis occurs in many disorders and results in a decrease in bicarbonate levels. The lungs compensate by hyperventilating.
   d. Nonrespiratory (metabolic) alkalosis is produced in many disorders and results in an increase in bicarbonate levels. The lungs compensate by hypoventilation.

7. The clinical significance of blood gas levels is as follows.
   a. pCO2 levels increase with administration of 100% oxygen or following exercise. Decreases (hypoxemia) indicate pulmonary difficulties, exposure to carbon monoxide, or improper anesthesia. Hypoxemia also may occur at high altitudes.
   b. pCO2 levels increase with respiratory acidosis. Decreased levels indicate respiration that is too rapid.

D. Nonprotein nitrogen measurements monitor and assess renal function. These substances arise from the breakdown of proteins and nucleic acids.
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1. Urea is the major excretory product of protein metabolism and is synthesized in the liver from carbon dioxide and ammonia arising from the deamination of amino acids. It is excreted by the kidney.
   a. All analytic methods include, in the initial step, the hydrolysis of urea by urease and subsequent production of ammonium. Most techniques are sensitive to excess ammonia contamination.
      (1) The coupled enzymatic assay is a kinetic assay that involves a second enzyme.
      (2) A pH indicator dye detects the presence of ammonium and causes a color change, which is read by a spectrophotometer.
      (3) The direct method does not use urease. It measures urea directly using diacetyl monoxime.
   b. Urea disorders typically involve an elevated level of urea in blood, which is referred to as azotemia. Disorders are named in association with the location of the dysfunction.
      (1) Prerenal azotemia is typically caused by decreased renal blood flow to the kidneys from congestive heart failure, shock, dehydration, decrease in blood volume, greater protein breakdown (as in major illness), or high-protein diet.
      (2) Renal azotemia is produced by renal failure.
      (3) Postrenal azotemia is caused by an obstruction anywhere in the renal system (e.g., tubules, ureter).
      (4) Uremia is a toxic condition involving a very high serum level of urea accompanied by renal failure.

2. Creatine is made in the liver from amino acids and used in muscle as an energy source. In its anhydrous state, it is called creatinine, which is excreted into the plasma in an amount proportional to muscle mass and then excreted in the urine. Because the level of creatinine is unaffected by diet, its level reflects the GFR.
   a. Analytic methods for creatinine
      (1) The Jaffé reaction involves the reaction of creatinine with picric acid to form a reddish chromogen. The absorbance is measured colorimetrically.
      (2) The kinetic reaction uses various enzymes and hydrogen peroxide to form a colored product.
      (3) Creatinine clearance is an estimate of the GFR obtained by measuring plasma creatinine and its rate of excretion into urine. This test requires a 24-hour urine specimen and blood sample for serum creatinine determination. The formula for calculation is:
      \[
      \text{UV} \times \frac{1.73}{A} 
      \]
      with urinary creatinine as \( U \) (mg/L), \( V \) as volume of urine (mL/min), \( P \) as plasma creatinine (mg/L), and \( 1.73 \) as normalization factor for body surface area.
   b. Abnormal creatinine levels are typically elevated because of abnormal renal function, such as reduced GFR. Creatinine levels are examined in conjunction with urea to determine the cause of azotemia. The normal blood urea nitrogen (BUN): creatinine ratio is 10 to 20:1. Higher ratios indicate that the elevation of BUN is caused by prerenal rather than renal causes.

3. Uric acid is synthesized in the liver from the breakdown of nucleic acids (DNA and RNA) and transported to the kidney for resorption.
   a. Analytic methods are based on the same initial reaction involving the oxidation of uric acid by uricase to allantoin and hydrogen peroxide. There are two coupled enzymatic methods that involve either:
      (1) The measurement of peroxide production following reaction with phenol and 4-amino phenazone (4-AP)
      (2) The measurement of peroxide production by the catalyzed oxidation of ethanol coupled to the production of acetate
b. Abnormal uric acid levels are either:
   (1) Elevated because of gout, increased nuclear breakdown (due to increased cell destruction, as in chemotherapy), renal disease, or toxemia of pregnancy
   (2) Decreased primarily because of severe liver or kidney disease

4. Ammonia is formed by the deamination of amino acids. It is used by the liver to produce urea and is not excreted by the kidney.
   a. Analytic methods include:
      (1) The coupled enzymatic method, in which ammonium reacts with α-ketoglutarate and reduced nicotinamide adenine dinucleotide phosphate (NADPH) to form L-glutamate and nicotinamide adenine dinucleotide phosphate (NADP), with changes in absorbance measured spectrophotometrically
      (2) The ammonia electrode, which is based on diffusion of NH₃ through a selective membrane
   b. Increased ammonia levels are typically caused by severe liver dysfunction (e.g., liver failure, Reye’s syndrome) or inadequate blood circulation through the liver.

E. Carbohydrates are polyhydroxyl aldehydes or ketones that, on hydrolysis, yield one of these compounds. Carbohydrates are a major source of energy for the body, and starch is the major source of carbohydrate.

1. Classification is based on the structure of carbohydrates.
   a. Monosaccharides are simple sugars that contain four to eight carbons and only one aldehyde or ketone group. These are reducing sugars (i.e., they can give up electrons). Examples include glucose and fructose.
   b. Oligosaccharides are formed by the interaction of two monosaccharides with the loss of a water molecule and are sometimes referred to as disaccharides. Examples include maltose, lactose, and sucrose.
   c. Polysaccharides are formed by interactions between many units of simple sugars. Examples are starch and glycogen.

2. Carbohydrate metabolism begins in the mouth.
   a. Salivary amylase breaks down ingested starches into disaccharides, and these are further broken down into monosaccharides by disaccharides and absorbed into intestinal cells.
   b. Monosaccharides are then transported to the liver and converted to glucose. Some glucose is released into the blood, and the rest is stored as glycogen in the liver and skeletal muscle.
   c. Glycogenesis is the process of glycogen formation by enzyme action on glucose to eventually form glycogen.
   d. Glycogenolysis is the breakdown of glycogen, with the eventual formation of glucose-6-phosphate or free glucose that can be used for energy production.
   e. Glycolysis is the catabolism of glucose to pyruvate or lactate for adenosine triphosphate (ATP) production (Embden-Meyerhof pathway and Krebs’ cycle).
   f. Gluconeogenesis is the formation of glucose from amino acids and lipids that occurs when carbohydrate intake decreases.

3. Factors that affect glucose levels include:
   a. Insulin, which is a pancreatic hormone that decreases glucose levels by increasing cellular uptake of glucose and promoting glycogenesis and lipogenesis (formation of fat from carbohydrates)
   b. Glucagon, which is a pancreatic hormone that increases glucose levels by stimulating glycogenolysis and gluconeogenesis
   c. Epinephrine, which is an adrenal hormone that elevates glucose levels
   d. Growth hormone and adrenocorticotrophic hormone (ACTH), which are pituitary hormones that increase glucose levels
   e. Glucocorticoids (e.g., cortisol), which are adrenal hormones that increase gluconeogenesis and eventually elevate blood glucose
   f. Thyroid hormones, which stimulate glycogenolysis and increase blood glucose levels
4. Glucose disorders depend on serum glucose levels.
   a. Hyperglycemia occurs when the fasting blood sugar level rises higher than 110 mg/dL due to a pathologic disorder, such as diabetes mellitus or liver failure.
   b. Hypoglycemia occurs when the fasting blood glucose level is <70 mg/dL. This typically occurs as a result of hormone deficiency, drug reaction, insulin excess (as in insulinoma), or a genetic disorder.
   c. Glycosuria (sugar in the urine) occurs when the renal threshold for glucose is exceeded (160–180 mg/dL) during hyperglycemia.
   d. Diabetes mellitus is a genetic disorder of glucose metabolism that results in insulin deficiency and lack of carbohydrate tolerance. There are two classifications:
      1. Type 1, formerly insulin-dependent diabetes mellitus. The individual presents in an acute state with hyperglycemia and ketosis. This type is caused by an autoimmune destruction of pancreatic $\beta$ cells and is usually juvenile onset. However, onset is often around the age of 20.
      2. Type 2, formerly non-insulin-dependent diabetes mellitus. This is a multifactorial disease with possible causes including genetics, environment (lifestyle), and autoimmunity. One cause of onset is thought to be cellular resistance to insulin or decreased insulin receptors on cell surfaces. Most individuals with this disorder are obese or overweight, and onset is typically during adulthood.

5. Methods of glucose analysis include:
   a. Glucose oxidase methods. Glucose is oxidized to gluconic acid and hydrogen peroxide to eventually form a colored product. Falsely low results are caused by high serum levels of uric acid, bilirubin, or ascorbic acid.
   b. Hexokinase methods. Glucose becomes phosphorylated and dehydrogenated to eventually form NADPH.
   c. o-Toluidine (nonenzymatic method). o-Toluidine reacts with glucose in acetic acid to form a colored product. Falsely elevated glucose values are obtained by interference of mannose and galactose, whereas bilirubin induces a false decrease in glucose values.
   d. Glycated hemoglobin methods. The presence of glycated hemoglobins is examined in diabetic patients. This test examines a patient’s compliance with an insulin therapy regimen over a period of 8 to 10 weeks. Glycated hemoglobins are formed when a glucose is attached to the N-terminal valine residue of either $\beta$ chain of the hemoglobin A molecule. Hemoglobin A$_1$ (made up of several minor hemoglobins) is termed a fast hemoglobin based on its electrophoretic migration. Glycated hemoglobin is separated from nonglycated hemoglobin by affinity chromatography. Interferences include the presence of hemoglobin S and hemoglobin F and may produce increased or decreased glycated hemoglobin values depending on the specific methodology used.

6. Glucose metabolism tests examine a patient’s ability to metabolize glucose.
   a. Glucose tolerance test (GTT) evaluates the insulin response challenge. It is useful in evaluating pregnancy-induced diabetes and involves drawing a fasting blood specimen, followed by patient ingestion of a 75-g oral dose of glucose in liquid within a 5-minute period. Blood samples are then taken at 30-, 60-, 120-, and 180-minute intervals and tested for glucose. Urine is tested as well.
      1. Nondiabetics have negative urine tests and show highest glucose levels at 30 to 60 minutes, with low and normal levels following.
      2. Severe diabetics reach peak glucose levels after 30 to 60 minutes, and the levels remain elevated.
   b. A 2-hour postprandial blood glucose test evaluates diabetes. A fasting blood specimen and a specimen taken 2 hours after breakfast are taken. Normal patients have no increase in serum glucose after 2 hours.

F. Lipids are substances that are insoluble in water and can be extracted from cells only by organic solvents (e.g., ether or chloroform). Lipids include fats (most abundant), steroids,
and terpenes. Fats are carboxylic esters derived from glycerol and are also known as glycerides.

1. Lipid composition of food
   a. Triglycerides comprise 98% of fat found in food and are made up of 95% fatty acid and 5% glycerol. Fatty acids are long carbon chains joined by single (saturated) or double bonds (unsaturated) and a terminal carboxyl group.
   b. The remaining 2% of fat in food is composed of cholesterol, phospholipids, diglycerides, fat-soluble vitamins, steroids, and terpenes.

2. The physiology of lipids involves three phases.
   a. Digestive phase begins with chewing and swallowing. Triglycerides are digested by lipase, other enzymes, bile salts, and acid in the gut to form monoglycerides and diglycerides. Cholesterol becomes surrounded by bile to form a micelle package that is absorbed by the small intestine.
   b. Absorptive phase occurs in the small intestine as triglycerides and cholesterol in the micelles are absorbed and broken down into fatty acids.
   c. Transport phase occurs as long fatty acids reassemble into chylomicrons (water-soluble macromolecules) and enter the lymphatic system. Short fatty acids enter the blood bound to albumin, and these head to all tissues, including adipose tissue.

3. Specific lipid physiology determines the function of each lipid.
   a. Cholesterol is a sterol (steroid with long side chains), which is a four-ringed structure made in liver hepatocytes from two acetate units. The process is long, and 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase is the committed step. Cholesterol is an important constituent of cell membranes and a precursor of many hormones. Most serum cholesterol is in the form of cholesterol esters, which are transported through the blood by low-density lipoproteins (LDL) and high-density lipoproteins (HDL).
   b. Triglyceride is made up of fatty acids and glycerol and is partly synthesized in the liver hepatocyte. It is transported through the bloodstream by chylomicrons and very low-density lipoproteins (VLDLs). Triglyceride provides energy to cells as it loses its fatty acid and forms ATP, thus acting as an energy store in the form of fat, and it insulates organs through fat deposits.
   c. Phospholipids make up the bilayer of cell membranes and also form a coating that surrounds cholesterol and triglyceride and “glues” them to the lipoprotein core.
   d. Sphingolipids are important in cell membrane composition and in nerve transmission.

4. Lipoproteins are transport vehicles for lipids that contain varying amounts of specific lipid, phospholipid, and apoprotein.
   a. Chylomicrons are large molecules that contain mostly triglyceride. They originate in the intestinal tract and travel through the blood and lymph to various tissues. They are degraded in the liver.
   b. VLDLs are smaller than chylomicrons. They contain mostly endogenous triglyceride, are made in the liver, contain equal amounts of phospholipids and cholesterol, and degrade to LDLs in the circulation.
   c. LDLs contain mostly cholesterol, with equal amounts of phospholipid and protein and some triglyceride. They are taken into cells via a special cell-surface receptor—the apoprotein B (apoB) receptor—and are degraded into component parts. This is considered “bad” cholesterol.
   d. HDLs contain mostly protein, some cholesterol, and a little triglyceride. They are made in the liver, and they remove excess cholesterol from cells. HDL is considered the “good” lipoprotein.

5. Lipid disorders usually lead to abnormal lipid deposits on walls of vasculature (atherosclerosis) and skin (xanthomas). Hyperlipidemia obstruction leads to lack of bile, so cholesterol cannot be adequately absorbed by the small intestine.
   a. Hyperlipidemia usually lead to abnormal lipid deposits on walls of vasculature (atherosclerosis) and skin (xanthomas). Hyperlipidemia obstruction leads to lack of bile, so cholesterol cannot be adequately absorbed by the small intestine.
     (1) Triglyceride levels are most affected by diet, but high triglyceride levels are often caused by diabetes or pancreatitis. Lipoprotein lipase (LPL), present in the capillary wall, hydrolyzes triglycerides for use in the tissues and can
Table 1–3 Fredrickson's Classification of Hyperlipoproteinemias

<table>
<thead>
<tr>
<th>Type</th>
<th>Plasma Appearance</th>
<th>Cholesterol Level</th>
<th>Triglyceride Level</th>
<th>Lipoprotein Abnormality</th>
</tr>
</thead>
<tbody>
<tr>
<td>I (rare)</td>
<td>Creamy</td>
<td>↔</td>
<td>↑↑↑</td>
<td>High chylomicrons</td>
</tr>
<tr>
<td>IIA (common)</td>
<td>Clear</td>
<td>↑↑↑</td>
<td>↔</td>
<td>High LDL</td>
</tr>
<tr>
<td>IIB (common)</td>
<td>Clear</td>
<td>↑↑↑</td>
<td>↑</td>
<td>High LDL, VLDL</td>
</tr>
<tr>
<td>III (rare)</td>
<td>Cloudy</td>
<td>↑↑</td>
<td>↑↑↑</td>
<td>Abnormal LDL, VLDL</td>
</tr>
<tr>
<td>IV (common)</td>
<td>Milky</td>
<td>↔</td>
<td>↑↑↑</td>
<td>High VLDL</td>
</tr>
<tr>
<td>V (rare)</td>
<td>Creamy; Milky</td>
<td>↑</td>
<td>↑↑↑</td>
<td>High chylomicrons, VLDL</td>
</tr>
</tbody>
</table>

↑ = slight increase; ↑↑ = moderate increase; ↑↑↑ = extreme increase; ↔ = no change; LDL = low-density lipoproteins; VLDL = very low-density lipoproteins.

be affected by various hormones. If LPL does not function properly, serum triglyceride levels rise.

2. Cholesterol levels are affected mostly by genetic defects in the liver or by lack of apoB receptors on cell surfaces, which leads to elevated cholesterol levels.

b. Hyperlipoproteinemia involves an increase in certain lipoproteins because of improper synthesis or breakdown of lipoprotein fractions. Hyperlipidemia can also induce overproduction of the lipoproteins. Fredrickson's classification types I to V (Table 1–3) is based on the appearance of plasma after 24 hours at 4 °C and on triglyceride and cholesterol values.

c. Hypolipoproteinemia is caused by a genetic defect leading to absent or decreased LDL and HDL.

1. Absent LDL and low serum cholesterol leads to a failure to thrive, steatorrhea, central nervous system degeneration, and malabsorption of fat and vitamins.

2. Decreased LDL leads to an increased life expectancy and decreased risk of myocardial infarction.

3. Reduced HDL leads to an increased risk of atherosclerosis.

4. Absent HDL (Tangier disease) leads to an accumulation of cholesterol esters in tonsils, adenoids, and spleen. It is considered a benign disease.

6. Methods of lipid analysis

a. Total cholesterol analysis involves either:

1. Formation of free cholesterol, which is oxidized to form hydrogen peroxide, which then reacts with a dye to form a colored product

2. Selective oxygen electrode, which measures the rate of oxygen consumption when an enzyme specific for cholesterol is added to serum

b. HDL cholesterol analysis involves precipitation of LDL and VLDL, followed by measurement of HDL in the supernatant.

c. LDL cholesterol analysis involves one of the following:

1. Calculation by the following formula:

\[
LDL = \text{total cholesterol} - \left( \frac{\text{HDL} + \text{triglyceride}}{2} \right)
\]

2. Ultracentrifugation

3. Immunoassay using an ag—ab reaction

d. Triglyceride analysis uses either:

1. Enzymatic method that involves three enzymes—lipase, glycerol kinase, and glucose-6-phosphate-dehydrogenase (G6PD)—to form NADH

2. Colorimetric method involving the formation of hydrogen peroxide

G. Vitamins are organic molecules required by the body in small amounts for normal metabolism. Most are obtained from the diet; some are formed endogenously; others are produced by the intestine.

1. Fat-soluble vitamins include A, D, E, and K.

a. Vitamin A has various forms: retinal, retinol, and retinoic acid. Most carotenoids found in vegetables are precursors of vitamin A. Most vitamin A is stored in
the liver, and some is transferred to cells to promote mRNA synthesis. The best understood physiology of vitamin A is in the visual system; a lack of vitamin A leads to night blindness.

b. Vitamin D is a sterol derivative known as cholecalciferol. It is produced in the skin by absorption of UV light or obtained by dietary intake and stored in adipose tissue. Vitamin D is essential for bone mineralization and neuromuscular activity, and some forms act to regulate uptake of calcium and phosphate. A lack of vitamin D leads to rickets (failure of bones to calcify) or osteomalacia (abnormal bone synthesis).

c. Vitamin E (a-tocopherol) is available only from dietary intake and accumulates in the liver, adipose tissue, and muscle. Vitamin E is critical for normal neuromuscular structure and function, and also functions as an antioxidant by preventing formation of free radicals. A deficiency of vitamin E produces a normocytic normochromic anemia.

d. Vitamin K is supplied partly by diet and partly by flora in the bowel. Vitamin K is known as phylloquinone and is essential in the formation of coagulation factors, particularly prothrombin (see Chapter 2, III D).

2. Water-soluble vitamins include vitamin C and the vitamin B complex.
   a. Vitamin C (ascorbic acid) is absorbed in the stomach and distributed to all tissues. It is a potent reducing agent, functions in the synthesis of collagen, and aids in the biosynthesis of some neurotransmitters. It is considered important in reducing the risk of certain cancers and the common cold.
   b. The B complex vitamins are grouped together because of their isolation from the same sources. Most B vitamins are known to serve as enzyme cofactors, functioning to transport atoms between molecules in enzyme-coupled reactions. Deficiency of any B vitamin affects the body’s metabolism. The B vitamins include thiamine, riboflavin, niacin, pantothenic acid, biotin, pyridoxine (B6), the folates, and cyanocobalamin (B12). Lack of vitamin B12 produces pernicious anemia because of its function in erythropoiesis. Deficient folate leads to a megaloblastic anemia, and in pregnant women, the possibility of fetal neural tube defects.

H. Tumor markers are substances that are synthesized and released by cancer cells or made by other tissues in response to the presence of cancer cells. These markers may be present in the blood, in other fluids, on cells, or within cells.

1. Clinically relevant (diagnostically accessible) tumor markers must have high disease sensitivity and specificity, and levels should reflect the status of the disease process. These analytes are typically examined by some form of immunosassay.
   a. Oncofetal antigens exist as normal proteins in the embryo and fetus and are also found in certain tumors.
      (1) Carcinoembryonic antigen (CEA) is an oncofetal antigen used to assess tumors of the colon. It is not as specific as once thought, and not all colon cancers have elevated CEA levels.
      (2) a-Fetoprotein (AFP) is an oncofetal antigen used to determine the presence of hepatic tumors (hepatoma) and testicular tumors.
      (3) Carbohydrate-associated antigen (CA-125) is a glycoprotein oncofetal antigen that appears in the serum of patients with ovarian cancer.
      (4) CA-19 is another glycoprotein oncofetal antigen associated with gastrointestinal tumors.
      (5) Prostate-specific antigen (PSA) is an oncofetal antigen that is important in screening and monitoring patients with prostate carcinoma.
      (6) Bladder tumor-associated antigens include fibrin degradation products and complement-related proteins.
   b. Placental proteins are synthesized by placental trophoblasts and certain tumors of the trophoblast.
      (1) Chorionic gonadotropin (CG) is typically used to determine pregnancy; however, high levels can indicate tumors of the testes or ovaries, as well as trophoblastic neoplasia.
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(2) Human placental lactogen (hPL) is used to monitor fetal well-being, but its levels also can be elevated in patients with trophoblastic neoplasms.

c. Certain enzymes elevations can indicate the presence of cancer cells such as alkaline phosphatase for bone cancer.

d. Increased levels of some hormones indicate malignancy of the organ that produces the hormone. For example, extremely elevated growth hormone levels might indicate a pituitary tumor.

2. DNA analysis determines the proliferative capacity of tumor cells including those involved in leukemia. Molecular diagnosis is used to identify oncogenes, the genes that transform normal cells into malignant cells. This type of testing is becoming more widespread with the advent of simplified molecular techniques. See Chapter 10 for details.

I. Porphyrin is a chemical intermediate in the synthesis of hemoglobin, myoglobin, and other respiratory pigments called cytochromes. Porphyrin measurement is important for the determination of porphyria (a disturbance of heme synthesis). Heme synthesis begins in the mitochondria.

1. Porphyrin chemistry involves the following principles.

a. Synthesis. Porphyrin is synthesized from porphin (four pyrrole rings), but side chains are substituted for the eight hydrogen atoms. A variety of substances make up the side chains (magnesium makes up part of the side chains in chlorophyll, which is a porphyrin).

(1) The main sites of porphyrin synthesis are bone marrow cells and liver cells.

(2) The rate of synthesis is controlled through regulation of the enzyme δ-aminolevulinic acid (ALA) synthase by the hemoproteins in the liver.

b. Clinical importance

(1) Three porphyrin compounds are clinically important: protoporphyrin (excreted in feces), uroporphyrin (excreted in urine), and coproporphyrin (excreted in both). All are intermediate products in the synthesis of heme that can be assessed clinically.

(2) Porphobilinogen (PBG) and ALA are precursors of porphyrin and can accumulate in certain porphyrin disorders. These substances are typically found in the urine of patients with acute porphyria.

(3) Free erythrocyte porphyrins (FEP) are porphyrins that can be extracted from RBCs, the primary one being protoporphyrin. FEP concentration is increased in persons with lead poisoning and iron deficiency anemia.

2. Porphyrrias are inherited or acquired disorders of heme synthesis in which overproduction of heme precursors in the bone marrow or the liver cause characteristic symptoms. The porphyrias are classified based on the signs and symptoms manifested by the patient (neurologic versus cutaneous). The primary cause of porphyrias is a specific enzyme deficiency.

a. The neurologic porphyrias include the symptoms of abdominal pain, psychotic behavior, and neuromuscular difficulties. Clinically, the three neurologic porphyrias have in common increased urinary ALA and porphobilinogen levels.

(1) Acute intermittent porphyria (most common) has a clinical presentation of increased uroporphyrin levels.

(2) Variegate porphyria (rare) has a presentation of increased protoporphyrin and coproporphyrin levels.

(3) Coproporphyria (rare) has a presentation of increased coproporphyrin levels.

b. The cutaneous porphyrias are induced by the presence of excess porphyrins in the skin, which generate oxygen radicals that attack cells and produce photosensitivity and skin lesions. The three cutaneous porphyrias have in common normal urinary ALA and porphobilinogen levels.

(1) Congenital erythropoietic porphyria (the rarest of the inherited porphyrin disorders) is a severe disorder that has a presentation of increased uroporphyrin and coproporphyrin levels.
(2) Protoporphyria (somewhat rare) has a presentation of increased protoporphyrin and increased free erythrocyte protoporphyrin levels.

(3) Porphyria cutanea tarda (most common) appears in adults following liver disease or excessive alcohol intake. It has a clinical presentation of increased levels of uroporphyrin.

c. Porphyrinuria is a moderate elevation of urine coproporphyrin secondary to a number of disorders, including pregnancy, neoplasia, intoxication, and liver disease.

d. Porphyrinemia is a moderate elevation in erythrocyte protoporphyrin secondary to a number of disorders, including:
   (1) Iron deficiency states, caused by poor nutrition, malabsorption, poor iron transport, or blood loss
   (2) Anemia (hemolytic, iron-deficiency, sideroblastic)
   (3) Lead poisoning

3. Methods of porphyrin analysis include qualitative and quantitative procedures. Specimen collection techniques are important. All samples must be protected from light; urinary porphyrin concentrations can decrease by up to 50% if kept in the light for 24 hours. Urinary porphyrins and PBG are best analyzed in fresh, early morning (10–20 mL) urine specimens collected without preservative.

   a. Qualitative tests include those for:
      (1) Urine porphobilinogen, which is a screening test whereby normal results are negative (e.g., the Watson-Schwartz test, the Hoesch test)
      (2) Urine porphyrins, which is a screening test performed to measure uroporphyrin and coproporphyrin

   b. Quantitative tests include a variety of procedures:
      (1) Urine tests for presence of porphobilinogen, uroporphyrin, coproporphyrin, and ALA
      (2) Blood tests include analysis of ALA dehydratase (an enzyme in the blood that breaks down ALA) and whole blood or erythrocyte protoporphyrin
      (3) Fecal tests for coproporphyrin and protoporphyrin

J. Iron is contained in the porphyrin ring of heme in hemoglobin, myoglobin, catalase, and cytochromes. The reversible interaction of iron with oxygen and the ability of iron to function in electron transfer reactions make iron physiologically important. Bilirubin is an orange-yellow pigment derived from aged RBC and formed in the spleen. It is transported to and transformed in the liver and is excreted in bile and urine.

1. Iron metabolism is controlled by its absorption in the intestine. Most circulating iron is derived from the release of iron following RBC destruction.

   a. Absorption of iron occurs mainly in the duodenum and includes only 5% to 10% of daily iron intake. The body’s iron supply is controlled by intestinal absorption.

   b. Iron transport involves a transport protein, transferrin, which binds ferric iron. The bound iron either moves to the mitochondria for heme synthesis or is stored in cells as ferritin.

   c. Iron storage includes storage as soluble ferritin (found in most cells) or relatively insoluble hemosiderin. One third of iron is stored in the liver, one third in the bone marrow, and one third in the spleen.

2. Iron disease states include:

   a. Iron deficiency anemia, which is usually caused by blood loss (menstrual cycle, ulcer, tumor)

   b. Iron overload, including:
      (1) Hemosiderosis (no tissue injury)
      (2) Hemochromatosis
         (a) Hereditary hemochromatosis is an autosomal recessive disease in which iron is deposited directly in the liver, heart, and kidney, leading to organ failure.
         (b) Sideroblastic anemia is caused by iron overload of unknown cause.
Table 1–4 | Iron-Binding Capacity (IBC) and Transferrin Levels in Specific Disease States

<table>
<thead>
<tr>
<th>Disease</th>
<th>Transferrin Level</th>
<th>Total IBC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iron deficiency anemia</td>
<td>High</td>
<td>High</td>
</tr>
<tr>
<td>Failure to incorporate iron into RBCs</td>
<td>Normal</td>
<td>Low</td>
</tr>
<tr>
<td>Iron overload</td>
<td>Normal</td>
<td>Low</td>
</tr>
</tbody>
</table>

(c) Acquired hemochromatosis follows thalassemias or lead poisoning. This disorder also occurs with chronic excessive absorption of normal iron intake.

3. Iron analysis involves the examination of three iron compartments: blood cells (hemoglobin), stored iron (ferritin), and circulating iron (i.e., serum iron, transferrin, iron-binding capacity (IBC)).
   a. The assay for measuring serum iron is based on the release of iron from transferrin and the subsequent reduction of ferric iron to ferrous iron. Serum iron is decreased during iron deficiency states, chronic inflammation, and menstruation; following blood loss; and following myocardial infarction. Iron is increased following overload, iron poisoning, and hepatitis, and during the use of oral contraceptives.
   b. IBC is a measurement of the maximum concentration of iron that transferrin can bind.
   c. Transferrin levels are analyzed by immunoassay techniques or radial immunodiffusion. Table 1–4 presents the IBC and transferrin results that occur in specific disease states.
   d. Plasma ferritin is the most sensitive indicator of iron deficiency. Ferritin levels decline early in anemia and increase early in chronic diseases.

4. Bilirubin synthesis involves catabolism of protoporphyrin IX to form biliverdin which is reduced to bilirubin. Bilirubin is then bound to albumin and transported to the liver. Bilirubin then dissociates from albumin at the membrane of the hepatocyte. It is then transported across the membrane and rapidly conjugated with glucuronic acid. In the intestine bilirubin glucuronides are hydrolyzed and is then reduced by intestinal microbial flora to form urobilinogen. Some urobilinogen is reabsorbed into the liver and excreted as bile, some circulates in the blood, and some is excreted in urine. Some is oxidized into urobilin and forms the bile pigments of stool. For disorders of bilirubin metabolism, see Section III B 2b.

K. Cardiac Markers are analytes that are utilized to assess the occurrence of myocardial infarction (MI) or the extent of damage produced by cardiovascular disease. These markers are proteins or enzymes (creatine kinase in particular) and display a variety of specificities and timelines of appearance.

1. Troponin (Tn) is a protein found in muscle tissue. There are three subunits of Tn (TnC, TnI, and TnT), two of which are useful for diagnosis and risk “stratifification” of MI: TnT and TnI. The three subunits are not structurally or even genetically related, but were named because they were once believed to be one protein.
   a. TnT and TnI are found in cardiac and skeletal muscle with a different gene encoding for the forms found in the two muscle types.
   b. TnC is less specific than the others, because the same amino acid sequence makes up this protein subunit in both skeletal and cardiac muscle tissue.
   c. Clinical utility of Tns is in the fact that the complex is tightly bound to the contractile apparatus of a muscle. When blood stops flowing to muscle tissue, there is degradation of the subunits and serum washes them from the cytosol. This leads to a peak of serum troponin T concentration within 24 hours of the MI (may increase between 1 and 6 hours post-AMI), peak at 10 to 24 hours, and return to normal after 10 to 15 days; TnT stays in serum longer than TnI.

2. Myoglobin is an oxygen-binding home protein present in cardiac and skeletal muscle and is relatively nonspecific. It increases within 2 hours post-AMI, peaks in
approximately 6 to 9 hours, and becomes normal in approximately 24 hours. Many things can increase serum myoglobin levels including strenuous exercise, surgery, renal failure, and muscular dystrophy. Myoglobin is quickly passed by the kidneys, and elevations of it in serum may be missed if blood is not drawn appropriately.

L. Bone is composed of two matrix components: minerals (calcium and phosphate) and an organic (collagen) component. The cellular components of bone include osteoblasts (bone-forming), osteocytes, and osteoclasts (responsible for bone resorption).

1. Bone disease can be divided into metabolic disorders, disorders of mineral metabolism, and disease of unknown etiology.
   a. Metabolic bone disease includes the following.
      (1) Osteoporosis is a decrease in the total amount of bone due to either decreased osteoblastic activity or increased osteoclastic activity.
      (2) Osteomalacia is the formation of “soft” bones due to inadequate calcification of matrix components. It is typically caused by vitamin D deficiency leading to hypocalcemia and elevated PTH.
      (3) Rickets is a hereditary disorder with symptoms similar to osteomalacia; in children, this includes bowing of the extremities. It is caused by phosphate depletion through renal wasting or by vitamin D deficiency or PTH excess.
      (4) Scurvy is caused by lengthy deficiency of vitamin C that leads to inadequate collagen synthesis and abnormal bone formation.
      (5) Renal osteodystrophy is renal failure that induces hyperphosphatemia, low vitamin D, etc. with osteomalacia as a complication. Any renal problem accompanied by a bone disorder fits into this category.
   b. Disorders of mineral metabolism include hyperparathyroidism, hyperpituitarism, hypoparathyroidism, hypophosphatemia, and hypomagnesemia. These are all discussed in previous lectures.
   c. Disorders of unknown etiology include Paget’s Disease (osteitis deformans) which is loss of bone mass followed by replacement of bone in a chaotic architecture. Bones are progressively enlarged and deformed. It is thought that a “slow virus” affecting osteoclasts is the cause.

2. Laboratory analysis of bone disease consists of several approaches.
   a. Mineral concentration in serum is analyzed, including calcium, phosphate, and magnesium. These values depend on bone deposition/resorption, renal clearance, and intestinal absorption.
   b. Hormone concentration in serum is analyzed, including PTH, vitamin D, and calcitonin. In addition, estrogen and testosterone are evaluated. In diagnosis of osteoporosis particularly, serum estrogen levels are important.
   c. Markers of bone metabolism, or bone markers, are of great importance when diagnosing bone loss. These include osteocalcin, alkaline phosphatase, urinary hydroxyproline, deoxypyridinoline, and N-telopeptide.
      (1) Markers of bone formation
         (a) Osteocalcin is a non-collagenous protein in bone that is synthesized by osteoblasts. Serum levels of this marker reflect osteoblast activity or bone formation/resorption. Synthesis of osteocalcin depends on vitamin K. Methods of detection include immunoassays.
         (b) Alkaline phosphatase (ALP) is elevated during bone growth, hyperparathyroidism, renal osteodystrophy, osteomalacia, estrogen deficiency, and Paget’s disease. It is synthesized in part by osteoblasts and is a good indicator of overall bone formation activity.
      (2) Markers of bone resorption
         (a) Urinary hydroxyproline is found in collagen fibers. When collagen is degraded, urinary excretion is increased. This marker is considered a marker for bone resorption. It is nonspecific, however.
         (b) Deoxypyridinoline is a protein that stabilizes collagen chains during collagen synthesis. It is considered a highly specific marker of bone formation. Techniques used to assay this marker include HPLC and immunoassay.
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(c) Urinary N-telopeptide is a marker that is released when osteoclasts degrade type I collagen that is found in bone. It is a highly specific marker of bone breakdown. This marker is assayed by ELISA.

(d) Acid phosphatase is released into blood during bone resorption.

V. ENZYMEOLOGY

A. Enzymes are proteins that catalyze biochemical reactions but do not alter the equilibrium point of the reaction. They are not consumed or altered. Enzymes are specific to each physiologic reaction of which they are a part.

1. Composition. Each enzyme is composed of a specific amino acid sequence (primary structure), which results in a steric arrangement (secondary structure) that becomes folded (tertiary structure).

2. Structure. Each enzyme contains an active site that binds a substrate and an allosteric site.

3. Cofactors may be necessary for enzyme activity and can be activators (inorganic) or coenzymes (organic). If the cofactor is bound to the enzyme, it is called a prosthetic group, and the enzyme portion is called an apoenzyme. The entire enzyme-cofactor molecule is called a holoenzyme.

4. Classification is based on the action of the enzyme.
   a. Oxidoreductases catalyze an oxidation-reduction reaction. Examples include lactate dehydrogenase and G6PD.
   b. Transferases catalyze the transfer of a group other than hydrogen. Examples are aspartate transaminase, alanine transaminase, creatine kinase, and γ-glutamyl transferase.
   c. Hydrolases catalyze the hydrolysis of ether and ester. Examples are alkaline phosphatase, acid phosphatase, amylase, and cholinesterase.
   d. Lyases catalyze the removal of groups without hydrolysis (loss of hydroxide ion).
   e. Isomerases catalyze the interconversion of geometric or optical isomers.
   f. Ligases catalyze the joining of two substrate molecules.

5. Enzyme kinetics deal with the relationship between the enzyme, the substrate, and the product.
   a. The catalytic mechanism is stated as:

      \[
      E + S \rightarrow ES \rightarrow E + P
      \]

      The transition state for the ES complex has a lower energy of activation than S alone, so the reaction proceeds after the ES complex is formed.

   b. Michaelis-Menten constant (\(K_m\)) expresses the relationship between the velocity of any enzymatic reaction and the substrate concentration (Figure 1–4). \(K_m\) is the substrate concentration at which the enzyme yields half the possible maximum velocity of the reaction.

   \[V = \frac{V_{max}[S]}{K_m + [S]}\]

   (1) The Michaelis-Menten hypothesis of the relationship between reaction velocity and substrate concentration is expressed as a formula:

   ![Figure 1–4 Michaelis-Menten curve.](image-url)
Figure 1–5 Lineweaver-Burk plot. $K_m = \frac{1}{V_{max}}$, $[S]$ = concentration of substrate; $V_{max}$ = maximum velocity.

(2) A Lineweaver-Burk plot expresses $K_m$ as a straight line, in which $V_{max}$ is the reciprocal of the $y$ intercept of the straight line, and $K_m$ is the negative reciprocal of the $x$ intercept of the same line (Figure 1–5).

c. Factors that influence enzymatic reactions include:

1. Substrate concentration, by following either:
   a. First-order kinetics, in which the reaction rate is directly proportional to the substrate concentration. With enzyme excess, the reaction rate steadily increases as more substrate is added until the substrate saturates all available enzymes.
   b. Zero-order kinetics, in which the reaction rate is dependent on enzyme concentration only. When product forms, the excess enzyme combines with excess free substrate.

2. Enzyme concentration, when exceeded by substrate concentration, causes the velocity of the reaction to be proportional to the enzyme concentration.

3. pH. Each enzyme operates maximally at a specific pH.

4. Temperature. Increased temperature increases the rate of a chemical reaction by increasing the movement of molecules.

5. Cofactor concentration. Increasing the cofactor concentration increases the velocity of an enzymatic reaction similar to substrate concentration.

6. Inhibitors
   a. Competitive inhibitors bind to the active site of the enzyme, causing $K_m$ to increase.
   b. Noncompetitive inhibitors bind at a place other than the active site, causing $V_{max}$ to decrease.
   c. Uncompetitive inhibitors bind to the ES complex; both $V_{max}$ and $K_m$ decrease.

d. Enzyme activity can be measured as either an increase in product concentration, a decrease in substrate concentration, a decrease in coenzyme concentration, or an increase in concentration of altered coenzyme.

1. Endpoint measurements are performed after a reaction proceeds for a designated length of time, then is stopped. Measurement is made of the amount of reaction that has occurred.

2. Kinetic measurements are multiple measurements of absorbance change made at specific time intervals.

3. An international unit (U) of enzyme activity is the amount of enzyme that catalyzes the reaction of 1 $\mu$mol of substrate per minute under specific conditions (e.g., temperature, pH).
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B. Phosphatases promote the hydrolysis of phosphate esters.

1. Acid phosphatase (ACP) promotes the hydrolysis of orthophosphate esters, although the physiologic function of ACP is unknown.
   a. Almost every body tissue contains ACP, but significant levels are found in RBCs and platelets. In adult men, 50% of ACP is found in the prostate gland. High levels of ACP are found in semen as well.
   b. In the measurement of total activity typical substrates acted upon by ACP include p-nitrophenylphosphate or thymolphthalein monophosphate. All reactions to measure ACP are carried out at a pH < 6.0.
      1. Isoenzyme tests include:
         a) Inhibition of prostate ACP by tartrate, followed by the determination of the serum level of other ACP fractions
         b) Immunoassay with antibodies specific to certain isoenzymes
         c) Electrophoretic procedures
      2) Falsely low values are produced by improper anticoagulant (e.g., fluoride, oxalate, heparin) use, whereas hemolysis produces false elevations.
   c. The major diagnostic significance of ACP is its aid in detecting metastatic carcinoma. Other types of cancer and bone disease exhibit increases of enzyme.

2. Alkaline phosphatase (ALP) hydrolyzes phosphate esters, but the function of this enzyme is relatively unknown.
   a. ALP exists in a wide variety of tissues, with significant amounts found in liver, bone, intestine, kidney, and placenta.
   b. In the measurement of total activity, the Bessey-Lowry-Brock reaction using p-nitrophenylphosphate as the substrate for ALP is most commonly used with pH or reaction > 8.0.
   c. Improper anticoagulant use produces decreased values of ALP, and a hemolyzed specimen demonstrates a false increase in ALP value.
   d. The diagnostic significance of ALP is its elevation during the third trimester of pregnancy and in persons with liver disease (hepatitis, cirrhosis), bone disease, hyperthyroidism, or diabetes mellitus.
   e. There are four isoenzymes of ALP: bone, liver, intestinal, and placental fractions. In disease states, the fractions are affected as follows:
      1) The bone fraction levels are elevated in persons with bone disease (osteomalacia, rickets, Paget’s disease) and high in children.
      2) The liver fraction level is greatly elevated in persons with hepatobiliary obstruction.
      3) The placental fraction level becomes elevated between the 16th and 20th weeks of pregnancy.

C. Transaminases catalyze the transfer of amino groups and acids. This is an important reaction in the synthesis and degradation of amino acids.

1. Aspartate transaminase (AST) transfers an amino group between aspartate and keto acids (aspartate + α-ketoglutarate → oxaloacetate + glutamate). Pyridoxal phosphate (vitamin B6) is the coenzyme in this reaction.
   a. AST exists in cardiac tissue, liver, skeletal muscle, and RBCs.
   b. Three major approaches are taken in the measurement of total activity.
      1) The reaction with dinitrophenyldiazidine couples color reagent with keto acid product.
      2) The reaction with diazonium salts couples the salt with the keto acid product and forms a color.
      3) The coupled enzyme assay (Karmen method) involves NADH and malate dehydrogenase and keto acid to form NAD.
   c. The clinical significance of AST elevations is that the elevations occur in patients with myocardial infarction, viral hepatitis, and skeletal muscle disease.

2. Alanine transaminase (ALT) catalyzes the transfer of an amino group from alanine to α-ketoglutarate with the formation of glutamate and pyruvate.
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a. Distribution. ALT is localized in the liver, with some in the heart, skeletal muscle, and RBCs.

b. The measurement of total activity is similar to the reactions of AST, except that the coupled enzyme assay uses lactate dehydrogenase to reduce pyruvate to lactate and oxidize NADH.

c. The clinical significance of ALT is in the evaluation of hepatocellular disorders.

D. Creatine kinase (CK) catalyzes a reaction responsible for the formation of ATP in tissues, especially contractile systems. When muscle contraction occurs, ATP is hydrolyzed to ADP to produce chemical energy for the concentration process. CK is involved in the storage of high-energy creatine phosphate (creatine + ATP $\rightarrow$ creatine phosphate + ADP).

1. Distribution. CK is localized in skeletal muscle, brain, and cardiac muscle in addition to many other tissues.

2. The measurement of total activity includes the following assays.
   a. The Tanzer-Gilvarg assay involves the reaction stated previously coupled with other enzymes (pyruvate kinase and lactate dehydrogenase) to produce a change in absorbance when measured spectrophotometrically.
   b. The Oliver-Rosalki assay is the reverse reaction of the one stated previously, in which creatine is produced from creatine phosphate. The enzymes used include hexokinase and G6PD.
   c. Hemolysis must be avoided when analyzing because of the presence of adenylate kinase (AK) in RBCs. AK induces false elevations in CK assays. Because CK activity is unstable due to the oxidation of its sulfhydryl groups, cysteine is added to both of the previously mentioned assays.

3. The clinical significance of CK is in its elevation in muscle disorders. CK and one of its isoenzymes is a sensitive indicator of acute myocardial infarction and muscular dystrophy.

4. The structure of CK allows for the formation of three isoenzymes.
   a. CK-MM is the major form found in striated and cardiac muscle and normal serum. Hypothyroidism and intramuscular injections result in CK-MM elevations.
   b. CK-MB is localized in cardiac muscle. Elevated levels are indicative of ischemic heart disease. If the serum level of CK-MB is $>6\%$ of total CK, it is a highly specific indicator of myocardial damage. Following acute myocardial infarction, CK-MB levels rise within 48 to 72 hours, peak at 12 to 24 hours, and return to normal within 48 to 72 hours.
   c. CK-BB is present in small quantities in normal serum and appears if extensive damage to the brain occurs, but is present in many other tissues.
   d. Assays used for measurement of isoenzymes include electrophoresis, ion-exchange chromatography, and immunoassay.

E. Lactate dehydrogenase (LD) catalyzes the interconversion of lactic and pyruvic acids. It is a hydrogen-transfer enzyme that uses the coenzyme NAD.

1. Distribution. LD is widely distributed in all tissues, with high concentrations found in the heart and liver and low levels in RBCs, skeletal muscle, and kidneys.

2. Measurement of total activity is based on the detection of NADH in the assay approaches.
   a. The Wacker method uses the lactate-to-pyruvate reaction with formation of NADH.
   b. The Wroblewski-Ladue method employs the reverse reaction of the Wacker method and measures the decrease in absorbance as NADH is consumed.

3. The diagnostic significance of LD is in the diagnosis of cardiac, hepatic, skeletal muscle, and renal disease. Highest LD levels are seen in persons with pernicious anemia, viral hepatitis, cirrhosis, and crush injuries. An elevated LD means little without isoenzyme studies.

4. There are, in normal serum, five different isoenzymes of LD (LD1 to LD5), with each isoenzyme being composed of four protein subunits.
a. LD1 is localized predominantly in heart muscle and RBCs and constitutes approximately 25% of total LD.
b. LD2 localization is similar to LD1, but it also is found in the kidneys and comprises approximately 35% of total LD.
c. LD3 is found in lung, lymphocytes, spleen, and pancreas. It constitutes approximately 22% of total LD.
d. LD4 is found mostly in liver and skeletal muscle and comprises approximately 10% of total LD.
e. LD5 is similar to LD4 in both localization and portion of total.

f. **Testing methodologies** for isoforms include:
   (1) **Electrophoresis.** LD1 migrates most quickly toward the anode, followed by the others in numerical order. After myocardial infarction, LD1 concentration is greater than LD2, which presents as a “flipped pattern” in electrophoresis. This pattern is also observed with hemolyzed specimens.
   (2) **Immunoinhibition for the measurement of LD1 activity.** This procedure involves an antibody that binds all other isoforms, leaving LD1 to be assayed by chemical reaction.

F. **Miscellaneous enzymes** that are clinically significant include γ-glutamyl-transferase (GGT), amylose, cholinesterase, pseudocholinesterase, and 5′-nucleotidase.

1. **GGT** transfers a γ-glutamyl residue to an amino acid (transpeptidation). This function is involved in peptide and protein synthesis.
   a. **Distribution.** Tissue sources of GGT include kidney, brain, prostate, pancreas, and liver. Urine contains significant amounts of GGT.
   b. **Measurement of total activity** is by the Szasz assay. In this reaction, the substrate is γ-glutamyl-p-nitroanilide, with the release of p-nitroaniline.
   c. The **clinical significance** of GGT is as follows.
      (1) GGT levels are elevated in almost all hepatobiliary disorders or biliary tract obstructions as well as in patients taking enzyme-inducing drugs like warfarin, phenobarbital, and Dilantin.
      (2) GGT levels are often examined with ALP levels. If both GGT levels and ALP values are high, some type of liver disorder is suspected. If GGT is normal and ALP is high, bone disease is likely present.
      (3) Patients with acute pancreatitis exhibit increased GGT levels. In persons with diabetes, GGT is typically increased as triglyceride levels rise.
      (4) Because alcohol has enzyme-inducing properties, the most useful application for GGT is in the detection of alcoholism and the monitoring of alcohol intake by patients during treatment.

2. **Amylase (AMS)** is a hydrolase that catalyzes the breakdown of starch and glycogen and produces products consisting of glucose, maltose, and dextrins. AMS is the smallest in size of all enzymes.
   a. **Distribution.** Tissue sources of AMS include the pancreas and salivary glands, as well as skeletal muscle.
   b. The **clinical significance** of AMS is its use in the diagnosis of acute pancreatitis. AMS levels rise 2 to 12 hours after onset of an attack and peak at 24 hours. Salivary gland lesions (e.g., mumps, parotiditis) also produce high levels of AMS. Patients with hyperlipidemia have low-to-normal AMS levels. Opiates constrict the pancreatic sphincters, which causes false elevations of AMS.

3. **Cholinesterase** is an enzyme that hydrolyzes the esters of choline. There are two types: acetylcholinesterase (AChE) and pseudocholinesterase (PChE). The substrate for AChE is acetylcholine (a neurotransmitter). PChE acts on many different substrates, but mainly butyryl esters.
   a. **Distribution.** AChE is found in RBCs, brain, and nerve cells. PChE is found mostly in the serum, liver, pancreas, heart, and white matter of the nervous system.
   b. The measurement of total activity includes:
      (1) **Manometric techniques** measure liberated carbon dioxide from the formation of acetic acid.
(2) **Electrometric measures** determine enzyme activity by measuring the pH decrease resulting from the liberation of acetic acid.

(3) **Photometric method (Ellman technique).** The substrate is a thiol ester that produces a thiol, which reacts with a disulfide to form a colored compound.

c. Regarding the **diagnostic significance** of the cholinesterases, PChE is of greatest interest. When the rate of protein synthesis declines, PChE levels decline; this indicates developing hepatocellular disease, starvation, or burn injuries. Low levels of PChE can also indicate insecticide poisoning.

4. **G6PD** is an oxidoreductase that catalyzes the oxidation of G6P to 6-phosphogluconate, which is an important first step in the pentose-phosphate shunt of glucose metabolism.

a. **Distribution.** Tissue sources of G6PD include the adrenal glands, spleen, thymus, RBCs, and lymph nodes. In the RBC, G6PD functions to maintain NADPH in a reduced form to protect hemoglobin from oxidation and to prevent red cell hemolysis.

b. In the **measurement of total activity,** the formation of NADPH is measured colorimetrically.

c. **Diagnostic significance.** Clinically, deficiency of G6PD is an inherited sex-linked trait most common in African-Americans. Increases of G6PD are seen in persons with myocardial infarction and megaloblastic anemia.

5. **5′-nucleotidase (5′-NT)** originates in the liver or diseased bone. 5′-NT becomes elevated in liver disease, as does ALP, but 5′-NT does not increase in patients with bone disease. Serum levels of 5′-NT are more sensitive to liver cancer.

### VI. ENDOCRINOLOGY

A. A hormone is a chemical substance that is produced and secreted into the blood by an organ or tissue and has a specific effect on a target tissue located away from the site of hormone production. The collection of hormones, carrier proteins, and other components of these processes forms the endocrine system; hormones act in conjunction with the CNS to maintain the internal chemical conditions necessary for cellular function and emergency demands.

1. **Hormone classification.** Hormones are classified either by their tissue of origin or their structure.

a. **Hormone classification by tissue of origin** involves the production of hormones by certain tissues (Table 1–5). Some tissues produce “releasing factors” that act on another tissue to release certain hormones.

<table>
<thead>
<tr>
<th>Table 1–5</th>
<th>Tissues and the Hormones They Produce</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Tissue of Origin</strong></td>
<td><strong>Hormone(s) Produced</strong></td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>Thyrotropin-releasing hormone, corticotropin-releasing factor, other releasing factors.</td>
</tr>
<tr>
<td>Anterior pituitary</td>
<td>Thyroid-stimulating hormone, adrenocorticotropic hormone (ACTH), follicle-stimulating hormone, luteinizing hormone, prolactin, growth hormone</td>
</tr>
<tr>
<td>Posterior pituitary</td>
<td>Vasopressin, oxytocin</td>
</tr>
<tr>
<td>Adrenal medulla</td>
<td>Epinephrine, norepinephrine</td>
</tr>
<tr>
<td>Adrenal cortex</td>
<td>Cortisol, aldosterone, 11-deoxycortisol</td>
</tr>
<tr>
<td>Thyroid</td>
<td>Triiodothyronine, thyroxine, calcitonin</td>
</tr>
<tr>
<td>Parathyroids</td>
<td>Parathyroid hormone</td>
</tr>
<tr>
<td>Pancreas</td>
<td>Insulin, glucagon</td>
</tr>
<tr>
<td>Gastrointestinal tract</td>
<td>Gastrin, others</td>
</tr>
<tr>
<td>Ovaries</td>
<td>Estrogens, progesterone</td>
</tr>
<tr>
<td>Placenta</td>
<td>Progesterone, human chorionic gonadotropin, human placental lactogen</td>
</tr>
<tr>
<td>Testes</td>
<td>Testosterone, other androgens</td>
</tr>
<tr>
<td>Kidneys</td>
<td>1,25-(OH)2 vitamin D, erythropoietin</td>
</tr>
<tr>
<td>Unknown</td>
<td>Prostaglandins</td>
</tr>
</tbody>
</table>
b. **Hormone classification by structure** involves the specific chemical makeup of hormones. Hormones of the same basic structure appear to produce the same fundamental biochemical changes.

1. **Peptide hormones** make up the majority of hormones. **Releasing factors** are peptides. The hormones are water-soluble and do not require transport proteins to move through the blood. They are synthesized and stored within the cell and have a short half-life (5–60 minutes) in the circulation.

2. **Steroid hormones** are involved in the regulation of sexual development and characteristics. All steroid hormones are synthesized from cholesterol as the basic molecule. They are not water soluble, and they require a transport protein to travel through the blood. Steroid hormones are synthesized in the adrenal gland, gonads, or placenta and have a long half-life (60–100 minutes) in the circulation.

3. **Amino acid derivatives** (amines) have properties similar to those of both steroid and peptide hormones.

4. **Fatty acid derivatives** include the prostaglandins.

2. **Mechanisms of hormone action** typically depend on the type of hormone.

a. **Hormone/receptor interaction** involves the binding of hormone to a specific receptor molecule on or within a cell.

b. **Hormone binding** occurs on or within a cell.

1. **Binding at the cell surface** produces a conformational change in the receptor complex that leads to release of proteins and formation of AMP from ATP within the cell. **Cyclic AMP (AMP-c)** then causes cellular enzymes to be phosphorylated, and these enzymes can exert regulatory control on biochemical reactions within the cell. Most peptide hormones interact with cells in this manner. However, insulin phosphorylates without AMP.

2. **Internal binding** typically involves steroid hormones. Diffusion into the cell occurs before attachment to a specific receptor within the cell. The steroid/receptor complex then migrates to the DNA in the nucleus and exerts control over the type and amount of RNA produced and ultimately over what proteins are synthesized.

3. **Hormone release** is controlled by a number of factors.

a. **Regulation by releasing factors** involves a complex system of “factors” produced by tissues that induce synthesis of a specific hormone. For example, a decline in blood pressure causes corticotropin-releasing factor to be released, which in turn induces the release of adrenocorticotropic hormone and constriction of blood vessels.

b. **Prohormones** are precursors of peptide hormones. Following hydrolysis of a portion of its peptide chain, a prohormone becomes an active hormone. The hydrolysis is controlled by the cell containing the prohormone.

c. **Feedback control** involves the release of a hormone that regulated prior steps in the releasing process. For example, thyroid hormones feed back to the hypothalamus to “shut off” thyrotropin-releasing hormone so excess thyroid hormone will not be produced.

4. **Hormone transport proteins** affect both the concentration of a hormone and its influence on the system. Peptide hormones are transported in the unbound state.

a. **Types** of transport proteins are specific for each hormone. The attachment of hormone to protein is through noncovalent linkages.

1. **Steroid and thyroid hormones** are bound to albumin, sex hormone-binding protein, or cortisol-binding globulin.

2. **Amines** are transported by serum proteins and thyroxine-binding globulin.

b. **Transport protein level can affect hormone action.** Relative amounts of bound and free hormone determine the degree of stimulus provided by the hormone. Only the free (unbound) fraction of a hormone exhibits activity, so any situation that affects the transport protein level or degree of binding has an impact on the concentration of the free hormone. The measurement of the transport protein concentration is sometimes an integral part of the hormone assay.
B. The hypothalamus and pituitary gland are integral components of the endocrine system.

1. The pituitary gland is made up of two parts—the anterior lobe (adenohypophysis) and the posterior lobe (neurohypophysis)—and is located in a cavity at the base of the skull.
   a. The anterior pituitary cells are responsible for hormone production (Box 1–4).
   b. The posterior pituitary does not synthesize any known hormone but serves as a storage area for certain hormones (e.g., oxytocin, vasopressin) produced by the hypothalamus.

2. The hypothalamus is a part of the CNS that lies at the base of the brain above the pituitary. The hypothalamus is connected to the pituitary gland via a cluster of nerves and blood vessels called the pituitary stalk. Neurons in the hypothalamus produce a number of releasing and inhibiting factors that affect a number of other endocrine glands (Box 1–5).

3. The regulation of production and secretion of hormones that are produced or housed in the endocrine glands is explained by the concept of the feedback loop.
   a. The feedback loop is composed of two endocrine organs: the pituitary gland and the target endocrine gland.
   b. Two types of feedback exist.
      (1) Negative feedback occurs when the stimulating hormone induces production of a hormone, elevated levels of which turn off pituitary release of the stimulating hormone. For example, high levels of thyroid hormones stop the release of thyrotropin-releasing hormone (TRH) from the hypothalamus and thyroid-stimulating hormone (TSH) from the pituitary gland, which in turn halts production of the thyroid hormones.
      (2) Positive feedback occurs when a structure secretes a hormone in response to a stimulating hormone released from the pituitary gland. The released hormone induces more stimulating hormone to be released from the pituitary gland.

4. Pathologic conditions involving the pituitary or hypothalamus manifest themselves in a variety of symptoms.
   a. The clinical manifestations of anterior pituitary disorders result either from hypersecretion or hyposecretion of hormones.

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**Box 1–4** Hormones of the Anterior Pituitary Gland

- Prolactin (PRL)
- Growth hormone (GH)
- Luteinizing hormone (LH)
- Follicle-stimulating hormone (FSH)
- Thyroid-stimulating hormone (TSH)
- Adrenocorticotropic hormone (ACTH)

**Box 1–5** Hormones of the Hypothalamus

- Corticotropin-releasing factor (CRF)
- Gonadotropin-releasing hormone (Gn-RH)
- Growth hormone-releasing factor (GH-RF)
- Luteinizing hormone-releasing hormone (LH-RH)
- Oxytocin (stored here)
- Prolactin release-inhibiting factor (PIF)
- Prolactin releasing factor (PRF)
- Somatostatin (somatotropin-release inhibiting factor, SRIF)
- Thyrotropin-releasing hormone (TRH)
- Vasopressin (antidiuretic hormone; ADH; stored here)
Hypersecretion usually involves one hormone. It is not uncommon for hypersecretion to be associated with the hyposecretion of another tropic hormone.

(a) **Primary factors** center on disorders of the pituitary gland: either pituitary adenomas or pituitary hyperplasia.

(b) **Secondary factors** center on disorders of the hypothalamus or may relate to ectopic production of pituitary hormones by nonendocrine tumors or to the hyposecretion of hormone by the target tissue.

Hyposecretion can be decreased secretion of one hormone, a group of hormones, or all hormones. The latter condition is referred to as “panhypopituitarism.”

(a) Single hormone hyposecretion results from lesions on the hypothalamus.

(b) **Pituitary adenoma,** causing hypersecretion of one hormone, is commonly the cause of hyposecretion of remaining pituitary hormones because of the destruction of the pituitary gland by the growing tumor.

Specifics of pathologic conditions associated with dysfunction of the anterior pituitary gland.

(1) Growth hormone (GH)

(a) The effects of hypersecretion are age dependent. In adults, the resulting condition is called acromegaly, which is the progressive enlargement of the distal parts of the extremities. In children, the resulting condition is gigantism. Both conditions usually result from pituitary adenomas secreting GH and compression of adjacent tissues of the pituitary gland, causing hyposecretion of other tropic hormones.

(b) Hyposecretion in children leads to pituitary dwarfism (small stature but proportionally built).

(2) Prolactin (PRL)

(a) Hypersecretion causes galactorrhea or lactation and is associated with infertility and amenorrhea in women and impotence in men. It usually is induced by pituitary adenoma.

(b) **Hyposecretion** leads to the lack of lactation in postpartum women.

(3) ACTH

(a) Hypersecretion is referred to as Cushing’s disease, with symptoms including truncal obesity, hyperglycemia, hypertension, and protein wasting. It is caused by pituitary adenoma, adrenal hyperplasia, or excess production by a nonendocrine tumor.

(b) Hyposecretion causes weight loss, weakness, and gastrointestinal problems.

(4) TSH

(a) Hypersecretion causes thyrotoxicosis and is the result of either hyperactivity of the thyroid or hyperactivity of the pituitary gland, leading to increased TSH release.

(b) Hyposecretion is difficult to differentiate from primary hypothyroidism.

(5) Gonadotropins (FSH and LH)

(a) Hypersecretion results in sexual precocity and is usually a result of brain tumors in the region of the hypothalamus.

(b) Hyposecretion results in sexual underdevelopment and infertility.

c. The clinical manifestations of posterior pituitary dysfunction involve either vasopressin or oxytocin. These disorders result from either hypothalamic dysfunction or some peripheral disease.

(1) Anti-diuretic hormone (ADH, also known as Vasopressin) regulates water reabsorption and blood pressure by affecting the renal tubules and the arterioles.

(a) **Hypersecretion** of ADH results in a condition referred to as syndrome of inappropriate ADH secretion (SIADH). The disorder occurs in a wide variety of conditions, including meningitis, head injury, tuberculosis, hypoadrenalism, hypothyroidism, and cirrhosis. SIADH is associated with hyponatremia (low blood sodium) and hypertonic urine, despite normal
renal and adrenal function. Symptoms include weakness, malaise, poor mental status, convulsions, and coma. It is typically caused by release of ADH from ectopic tumors.

(b) **Hyposecretion** of ADH is associated with diabetes insipidus. Symptoms include insatiable thirst, polydipsia (excessive drinking), and polyuria (excessive urine volume). This disorder results from destruction of the posterior pituitary gland or the hypothalamus.

(2) No known disorders are associated with excess or deficient secretion of oxytocin.

C. **Adrenal glands.** One adrenal gland is situated on top of each kidney. Each adrenal gland is composed of two distinct layers: the adrenal cortex (the outer-most region) and the adrenal medulla (the innermost region).

1. **The adrenal cortex** is composed of three distinct tissues, the zona glomerulosa, the zona fasciculata, and the zona reticularis.

2. **The adrenal medulla** is composed of chromaffin cells.

3. **Adrenal hormones**
   a. **Glucocorticoids** are steroid hormones produced in the zona fasciculata and reticularis of the adrenal cortex.
   b. **Mineralocorticoids** are steroid hormones produced in the zona glomerulosa.
   c. **Catecholamines** are amine hormones produced in the adrenal medulla.

4. **Adrenal endocrine function** includes regulation of proteins, carbohydrates, and many other metabolic functions.
   a. **Glucocorticoids** are synthesized from cholesterol. They are transported bound to plasma proteins [albumin and corticosteroid-binding protein (CBP)], are catabolized by the liver and kidneys, and are regulated by the hypothalamus-pituitary-adrenal axis. The primary action of the glucocorticoids is catabolic (i.e., promoting protein and lipid breakdown, inhibiting protein synthesis).

   (1) **Cortisol** is the primary glucocorticoid produced and secreted by the adrenal cortex. Its functions include carbohydrate, lipid, and protein metabolism; suppression of inflammation; stimulating gluconeogenesis; increasing urine production; and stimulating erythropoiesis.

   (2) **Target tissues** of glucocorticoid action include the kidney glomerulus and renal tubules, bone marrow stem cells, hepatocytes, and adipose tissue.

   b. **Mineral corticosteroids** are synthesized from cholesterol, transported bound to CBP and albumin, and are catabolized by the liver and the kidney. The primary action is regulation of electrolytes.

   (1) **Aldosterone** is the primary mineralocorticoid produced and secreted by the adrenal cortex. Its functions include:

      (a) Stimulating sodium resorption in the distal convoluted tubules in exchange for potassium or hydrogen

      (b) Increasing blood volume (via renin/angiotensin system) and pressure

      (c) Regulating extracellular fluid volume

      (2) **Target tissues** of mineralcorticoid action include the distal renal tubules and the large intestine.

   (3) **Regulation of aldosterone secretion via the renin/angiotensin system** is achieved as follows. Decreased blood volume or blood pressure induces the release of kidney renin, which induces the production of angiotensin I and II. Angiotensin II affects release of aldosterone from the adrenal gland, which ultimately causes the kidney distal tubule to retain sodium, thereby raising blood volume and blood pressure.

   c. **Catecholamine** production is not limited to the adrenal medulla. Norepinephrine is predominantly synthesized in the CNS, whereas epinephrine is predominantly synthesized by the adrenal medulla. Catecholamines are products of the hydroxylation of the amino acid tyrosine. They are transported free in the blood and regulated by feedback inhibition of synthesis.
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A Concise Review of Clinical Laboratory Science

1. Catecholamine functions include:
   (a) Mobilization of energy stores by increasing blood pressure, heart rate, blood sugar level (by stimulating glycogenolysis)
   (b) Neurotransmitter actions
   (c) Release in response to pain and emotional disturbance (stress) to mobilize organs

2. Tissue targets include the liver and adipose tissue.

3. Approximately 20% of catecholamines are excreted into the urine as metanephrine and normetanephrine: approximately 80% are converted to vanillylmandelic acid (VMA) by the enzyme monoamine oxidase (MAO).

5. Diseases associated with the adrenal cortex center on hyperfunction (excess production of bioactive molecules) or hypofunction.
   a. Hyperadrenalism involves three basic conditions:
      (1) Cushing’s syndrome involves excess cortisol production, either at the level of the adrenal gland or by increased release of ACTH.
      (2) Hyperaldosteronism involves excess aldosterone production with symptoms of hypertension. There are two causes.
         (a) Conn’s syndrome is induced by an aldosterone-secreting adrenal adenoma or adrenal hyperplasia and is a rare cause of hypertension.
         (b) Excess renin production leads to elevated aldosterone levels.
      (3) Congenital adrenal hyperplasia is a genetic disorder causing a deficiency of enzymes in the synthetic pathways that lead to cortisol and aldosterone production. ACTH levels are increased, and steroid hormones are hypersecreted.
          A common cause of this condition is a 21-hydroxylase deficiency.
   b. Hypoadrenalism is caused by adrenal hypofunction or insufficiency and can be induced by three conditions:
      (1) Primary disease involving the adrenal cortex (known as Addison’s disease), which is relatively rare
      (2) Secondary adrenal insufficiency precipitated by decreased levels of corticotropin-releasing hormone (CRH) or ACTH
      (3) Long-term suppression of the hypothalamic-pituitary-adrenal axis by glucocorticoids

6. The major disorder of the adrenal medulla is pheochromocytoma. Pheochromocytoma is a relatively rare, usually benign tumor arising in the chromaffin cells of the adrenal medulla that results in hypersecretion of catecholamines.

7. Laboratory analysis of adrenal function involves several different testing methodologies.
   a. The laboratory investigation of Cushing’s syndrome proceeds in two stages.
      (1) The fact that the patient has autonomous cortisol production must be established by using the dexamethasone suppression test. This test involves the injection of a synthetic steroid that acts like cortisol (dexamethasone) to induce feedback inhibition of cortisol release at the level of the pituitary.
      (2) The cause of the disease must be differentiated.
   b. Plasma cortisol values normally display diurnal variation, with the highest levels occurring in the morning and the lowest levels in the early evening. Evening values are <50% of the early morning concentrations. Classically, samples are drawn at 8 a.m. and 4 p.m.
   c. Primary hyperaldosteronism is analyzed by examining serum and urine potassium. Values of serum potassium <3.5 mmol/L, coupled with a urine potassium excretion rate >30 mmol/24 hr, are commonly seen in persons with primary hyperaldosteronism. The definitive test for primary hyperaldosteronism, however, is the measurement of serum or urine aldosterone following a high salt challenge. Primary hyperaldosteronism caused by an aldosterone-producing tumor demonstrates no change in plasma aldosterone levels following salt challenge, whereas primary aldosteronism caused by adrenal hyperplasia demonstrates a rise in plasma aldosterone. A simple method for investigating Conn’s syndrome is to measure serum and urine potassium when a patient is not receiving diuretics.
d. Congenital adrenal hyperplasia is analyzed by investigating the possibility of adrenal insufficiency. The patient may also suffer biochemical consequences of excess androgen, and measurements of testosterone in serum and pregnanetriol in urine may be requested.

e. In the analysis of Addison’s disease, patients typically exhibit postural hypotension, hyponatremia, and hyperkalemia. The ACTH stimulation test is given; it involves injection of synthetic ACTH and subsequent measurement of ACTH and cortisol at 30 and 60 minutes. High ACTH with no cortisol response indicates Addison’s disease.

f. There are many ways to investigate pheochromocytoma. Urinary metabolites (e.g., VMA, metanephrines) can be quantitated, or plasma epinephrine and norepinephrine can be measured. In difficult cases, when the patient has episodic hypertension of short duration, a clonidine suppression test combined with plasma catecholamine measurements is performed. Clonidine suppresses catecholamine release from the CNS but not from the adrenal gland.

D. The thyroid gland is a bilobed endocrine gland located in the lower part of the neck that is composed of groups of cells called follicles. This gland contains two cell types: Follicular cells produce the hormones thyroxine (T4) and triiodothyronine (T3), and parafollicular cells (lying adjacent to the follicles) produce the hormone calcitonin.

1. Thyroid hormones require iodine for their synthesis. The iodine combines with the protein thyroglobulin to form hormone precursors that in turn combine to form T3 and T4.

a. The hormones are either stored within the follicle or released into the bloodstream. In the blood, most T4 eventually gives up an iodine molecule and forms T3. There is much more circulating T3 than T4.

b. Approximately 98% of circulating T3 and T4 is bound to protein, including thyroxine-binding globulin (TBG) and thyroxine-binding albumin. Some hormone remains unbound or free, and this is the physiologically active fraction.

c. Thyroid hormone function includes action at the cellular level to regulate carbohydrate, lipid, and protein metabolism. The hormones also act on the CNS, stimulate the heart, and have a role in physical growth and development.

d. The regulation of T3 and T4 occurs in the following manner.

(1) Thyroid-releasing hormone (TRH) is released by the brain and stimulates the release of TSH (thyrotropin) from the pituitary gland.

(2) TSH stimulates iodine uptake by the thyroid gland and also causes the release of T3 and T4 from the thyroid gland.

(3) High serum levels of free T3 and T4 “shut off” the release of TSH from the pituitary gland, whereas decreased levels induce TSH release.

2. Thyroid disorders are caused by increased or decreased levels of the circulating hormones T3 and T4. A wide variety of physical diseases can be traced back to a dysfunctional thyroid gland.

a. Hypothyroidism is a serum level of thyroid hormone that is insufficient to provide for the metabolic needs of cells. This disorder affects women four times more than men between the ages of 30 and 60 years. Hypothyroidism is usually referred to as primary, secondary, or tertiary, depending on the site of the dysfunction.

(1) The symptoms of hypothyroidism include an enlarged thyroid gland (goiter), fatigue, impairment of mental processes, and loss of appetite. Myxedema (loss of hair, swelling of the hands and face, course skin) occurs as the disease progresses.

(2) The causes of hypothyroidism relate to the area of tissue damage. In addition, hypothyroidism can be caused by lack of dietary iodine.

(a) Primary hypothyroidism involves the inadequate secretion of thyroid hormones caused by a damaged or surgically removed thyroid gland. Congenital hypothyroidism is caused by the absence of the thyroid gland. Laboratory results indicate decreased T3, T4, free thyroxine index (FTI), T3 uptake (T3U), and increased TSH.
(b) Secondary hypothyroidism involves decreased production of TSH caused by pituitary disorder leading to low serum levels of the thyroid hormones. Laboratory results indicate all thyroid test values are decreased.

c. Tertiary hypothyroidism is caused by hypothalamic failure leading to a lack of TRH production.

3. In the laboratory evaluation of hypothyroidism, the earliest abnormality is increased TSH, followed by decreased serum levels of T₄ and T₃.

b. Chronic immune thyroiditis (Hashimoto’s disease) is caused by a genetic abnormality in the immune system and involves massive infiltration of the thyroid gland by lymphocytes. The symptoms match those of hypothyroidism.

c. Hyperthyroidism is caused by excessive thyroid hormone in the circulation. This causes cells to become overactive. The disorder is sometimes referred to as thyrotoxicosis.

(1) The symptoms of hyperthyroidism include weight loss, loss of muscle mass, hyperactivity yet quick fatigability, insomnia, increased sweating, nervousness, palpitations, goiter, and bulging eyes.

(2) The causes of this disorder include pituitary tumors that cause excessive TSH secretion, thyroid carcinoma, or toxic multinodular goiter (gland produces excess hormones).

(3) The laboratory evaluation of hyperthyroidism in the initial evaluation reveals elevated thyroid hormone serum levels and decreased serum TSH.

d. Graves’ disease is an autoimmune disorder that occurs six times more frequently in women than in men. In this disorder, immunoglobulins stimulate the thyroid gland by binding to TSH receptors. Symptoms are similar to those of hyperthyroidism. Laboratory results indicate increased T₃, T₄, FT₄I, and T₃U, and decreased or normal TSH.

e. Thyroiditis is an inflammation of the thyroid gland caused by either bacterial or viral infection.

3. Assays for thyroid function include testing for serum level of total (both bound and free) or free T₄, total or free T₃, TSH, and TBG. These tests are typically immunoassays. Other thyroid tests include the following:

a. T₃ resin uptake analyzes the capacity of TBG to bind thyroid hormones. It is an indirect measurement of the number of free binding sites on the TBG molecule.

b. Free thyroxine index (FT₄I) indirectly assesses the concentration of circulating free T₄. It is calculated by multiplying the value of the total T₄ by the percentage value of the T₃ resin uptake.

c. Thyroid antibody screen assay for the presence of thyroid-stimulating immunoglobulins, such as those in Graves’ disease and Hashimoto’s thyroiditis.

d. TRH stimulation test measures pituitary TSH stores and is considered conclusive for hyperthyroidism, although it is not needed in most hyperthyroid patients. In patients with slightly elevated hormone levels (but other symptoms of hyperthyroidism), TRH is injected, and blood samples are assayed for TSH. TSH levels rise rapidly in a normal person but will not rise in a hyperthyroid patient.

E. Parathyroid hormone is produced by the parathyroid glands, which are small, paired structures located in the posterior thyroid capsule. They may, however, be located in other parts of the neck or upper chest cavity. The sole function of the parathyroid gland is the production of parathyroid hormone.

1. Parathyroid hormone (PTH) synthesis begins with the precursor proparathyroid hormone.

a. The major physiologic action of PTH is mineral homeostasis. Specifically, PTH is involved in the metabolism of both calcium and phosphorus by the kidney and bone. A complex interrelationship exists between PTH, cholecalciferol (vitamin D₃), and calcitonin.

b. Transport. Parathyroid hormone, unlike many other proteins, is transported as a freely circulating, intact, active molecule. The biologically inactive amino acid fragments can be detected in the serum as well.
c. The primary determinant of PTH release is the serum concentration of ionized calcium, considered to be the biologically active form of serum calcium. Other substances that impact PTH secretion rates include:
(1) Magnesium, which has an effect on PTH that both parallels and modulates the effect of calcium on PTH release
(2) Biogenic amines like epinephrine, dopamine, and serotonin
(3) Vitamin D

2. PTH has an important role in calcium and phosphorus metabolism.
   a. Calcium is a mineral proved to be essential for heart muscle contraction, hemostasis, and cell responsiveness. Calcium homeostasis is regulated not only by PTH but also by cholecalciferol (vitamin D) and the thyroid hormone calcitonin. The overall effect of PTH is to raise blood calcium levels through its action on bone and kidney.
   - In bone, PTH increases bone resorption of calcium.
   - In the kidney, PTH increases the renal reabsorption of calcium.

b. Phosphorus, or rather compounds of phosphate, is in all living cells and participates in many important biochemical processes. The overall effect of PTH is to lower phosphorus concentrations, whereas vitamin D acts to increase blood phosphate.

   c. Calcitonin is produced by parafollicular cells in the thyroid. In general, the overall effect of calcitonin is to decrease blood calcium because of its effect on both bone and renal calcium processing, which is just the opposite of PTH.
   - In bone, calcitonin inhibits bone resorption of calcium.
   - In the kidneys, calcitonin decreases the renal reabsorption of calcium, phosphorus, sodium, potassium, and magnesium.

3. Pathologic conditions involving a dysfunctional parathyroid gland most often manifest as hypocalcemic or hypercalcemic states.
   a. Hypocalcemia is classified as either being associated with deficient PTH concentration or being independent of PTH activity (vitamin D-deficient states). In general, the PTH-dependent hypocalcemic disorders fall into two categories: those related to hyposecretion of PTH and those related to tissue resistance to PTH (known as pseudohypoparathyroidism).
      - Primary hypoparathyroidism is synonymous with hyposecretion of PTH. It is caused by surgical removal of the parathyroid glands, trauma following surgery, or radiotherapy directed toward the thyroid gland. The clinical manifestations are those of hypocalcemia and include tetany (muscle spasms), skin changes (especially drying), brittle hair, hypotension, and GI upset. Low serum calcium and high phosphorus are hallmarks of this disorder.
      - Idiopathic hypoparathyroidism is rare and can be hereditary or seen in conjunction with other endocrine disorders. A very low serum calcium level and a very high phosphorus level are indicative of this condition.
      - Pseudohypoparathyroidism (PHP) is characterized by a lack of responsiveness to PTH by the renal system or other organ systems, not by a decrease in PTH. Serum PTH levels are typically normal to increased in this condition.
   b. Hypercalcemia is diagnosed when serum calcium levels rise to >102 mg/L or are sustained at levels >100 mg/L. Symptoms range from no symptoms to manifestations of polyuria, polydipsia, kidney stones, acid-base disorders, nausea, stupor, or coma. Malignancy (e.g., multiple myeloma, leukemia, lymphoma) and hyperparathyroidism account for most cases of hypercalcemia.
      - Primary hyperparathyroidism most often results from parathyroid adenoma or hyperplasia. Additionally, hyperparathyroidism can be associated with other endocrine disorders, collectively referred to as multiple endocrine neoplasia (MEN), which involve the pituitary, pancreas, thyroid, and adrenal glands. Serum calcium is increased, PTH is increased, and phosphorus is normal to decreased.
      - Secondary hyperparathyroidism is a condition associated with an attempt for the body to compensate for hypocalcemic states. It is commonly seen in patients with renal failure who cannot excrete phosphorus, resulting in a decrease in calcium, which stimulates the secretion of PTH. Serum calcium is low, PTH is increased, and phosphorus is increased.
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4. **Laboratory analysis of parathyroid function** involves determination of various hormone and electrolyte levels.

   a. The primary analytes of interest in parathyroid function evaluation are **calcium** (total and ionized), **phosphorus** (inorganic), and **PTH** (C-terminal, N-terminal). PTH levels should always be considered along with serum calcium levels.
   
   (1) **PTH C-terminal analysis** examines the intact PTH molecule and is highly specific for detecting hyperparathyroidism.
   
   (2) **PTH N-terminal analysis** measures both the whole PTH molecule and the amino-terminal fragments in the serum.

   b. Additional analytes of secondary interest include calcitonin, vitamin D, magnesium, bicarbonate, and nephrogenous cyclic adenosine monophosphate (cAMP).

F. **Reproductive hormones (Table 1-6).** A complex interrelationship exists between the hypothalamus, pituitary gland, and the gonads (ovaries and testes).

   1. **FSH and LH** are glycoprotein hormones synthesized in the anterior pituitary gland and transported unbound via the systemic circulation to target tissues.

   a. In a woman, FSH induces the growth of the ovum inside the follicle, and LH triggers release of the ovum.

   b. In a man, FSH induces spermatogenesis in the Sertoli’s cells of the testes, and LH stimulates the production of testosterone by the Leydig’s cells.

   2. Progesterone, testosterone, androgens, estrone, and estradiol are **steroid hormones** synthesized either in the testes, ovaries, or adrenal glands. Each of these steroid hormones has cholesterol as its precursor.

   a. The ovaries produce estrogens (i.e., estrone, estradiol), androgens, and progesterone. The estrogens are responsible for development of the uterus, fallopian tubes, and female sex characteristics. Estrogens also prepare the uterine endometrium (lining) for pregnancy. Progesterone prepares the breast for lactation and maintains the endometrium.

   (1) **Estradiol** is the chief estrogen produced specifically by the maturing follicle within the ovary.

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<th>Table 1-6</th>
<th>Reproductive Hormones</th>
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<td><strong>Endocrine Gland</strong></td>
<td>Hormone Produced/Released</td>
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<td>Hypothalamus</td>
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FSH = follicle-stimulating hormone; LH = luteinizing hormone
(2) Estrone, the other major biologically important estrogen, is produced either in the peripheral tissues via conversion of prohormone androstenedione or in the ovary from the conversion of estradiol.

(3) Another estrogen, estriol, is produced in the placenta. Therefore, little is present in nonpregnant women.

(4) Progesterone is produced by the corpus luteum (the structure that forms in a follicle that has released its ovum). It is present in significant amounts following ovulation.

(5) Androstenedione is the chief female androgen.

b. The testes in adult men produce testosterone and small amounts of androstenedione, dehydroepiandrosterone, and estradiol. Testosterone (the most potent of the androgens) induces growth of the male reproductive system, prostate gland, and development of male sex characteristics, including hypertrophy of the larynx and initiation of spermatogenesis. The other androgens are also responsible for the secondary sex characteristics in men.

c. Adrenal gland androstenedione is converted to testosterone in the peripheral tissues and accounts for approximately 5% of the total testosterone in men.

3. The regulation of all reproductive processes involves interrelated events and endocrine systems, including the hypothalamus, the pituitary gland, and the gonads.

a. In women, the menstrual cycle controls reproductive events. High serum levels of FSH induce follicular development and estrogen release. The increased level of estrogen causes a decrease in the release of FSH at the level of the pituitary gland and also causes an increase in serum LH. LH initially causes estrogen levels to decline, then induces maturation and rupture of the follicle, which releases ovum. Estrogen levels increase following this release, the ruptured follicle becomes the corpus luteum, and progesterone is produced. Progesterone inhibits LH release from the pituitary gland. Serum FSH and LH levels continue to decline, and regression of the corpus luteum induces decreased serum levels of estrogen and progesterone, initiating menstruation. The estrogen-induced inhibition of FSH release by the pituitary gland is removed, and FSH levels begin to increase, beginning the cycle again.

b. In men, increased levels of serum testosterone inhibit the release of LH from the pituitary, thereby decreasing the production of testosterone by Leydig’s cells.

4. Abnormalities of testicular function focus on the lack of or excessive production of androgens. Both increased and decreased androgen production can be a primary condition resulting from testicular dysfunction, or it may be a secondary condition resulting from hypothalamic-pituitary dysfunction.

a. The symptoms of hypogonadism are directly dependent on the time of the development of androgen deficiency. Prepubertal hypogonadism is caused by the absence of androgen production. Infantile genitalia persist; growth continues, but is decreased. This condition is often not apparent until the adolescent period when normal adolescent development does not occur. Postpubertal hypogonadism results in minimal changes.

(1) Primary hypogonadism is caused by a lack of androgenic feedback by FSH/LH on the hypothalamus-pituitary axis, which is typically caused by a genetic defect in testicular development. Laboratory results indicate increased serum and urine gonadotropins (LH/FSH), decreased serum androgens (testosterone), and decreased urinary 17-ketosteroids.

(2) Secondary hypogonadism is caused by primary hypopituitarism or a hypothalamic dysfunction that results in decreased production of LH and FSH by the pituitary gland. Absence of serum and urine gonadotropins and decreased serum androgens are indicated by laboratory results.

b. Hypergonadism is most often a condition resulting from excessive androgen production by a testicular tumor. It can also occur secondary to hypothalamic-pituitary dysfunction, with a resulting increase in FSH/LH secretion. In the adult, there is little physical change with these disorders. However, increased androgen levels in children result in precocious puberty.
Primary hypergonadism is indicated by high serum androgen levels, high urinary 17-ketosteroid levels, and low serum gonadotropin levels.

Secondary hypergonadism can be differentially diagnosed by the presence of high serum androgen levels, high urinary 17-ketosteroid levels, and elevated gonadotropin levels.

5. Abnormalities of ovarian function present as either hypo- or hyperfunction, both of which are considered as either primarily caused by ovarian disease or secondarily caused by hypothalamus and pituitary dysfunction.
   a. Ovarian hypofunction is also a time-dependent condition. If it occurs before the onset of puberty, it induces delayed or absent menstruation. If it occurs after the onset of puberty, it will result in secondary amenorrhea (lack of menstruation).
   (1) Primary ovarian hypofunction is caused by a lack of estrogenic feedback on the hypothalamus-pituitary system, which results in increased release of FSH and LH from the pituitary gland. Characterized by increased serum levels of gonadotropins and decreased estrogen levels, there are two common causes of this condition.
      (a) Menopause, the termination of reproduction in women
      (b) Turner’s syndrome, a congenital endocrine disorder in which the ovaries cannot secrete estrogen
   (2) Secondary ovarian hypofunction results from hypothalamic-pituitary dysfunction or serious illness. Laboratory results indicate decreased serum gonadotropins, estrogen, and progesterone. The three most common causes of this condition are:
      (a) Tumors and necrosis of the pituitary gland (e.g., Sheehan’s syndrome)
      (b) Congenital hypothalamic disorders
      (c) Illnesses such as congenital heart disease, chronic renal disease, rheumatoid arthritis, rapid weight loss, anorexia nervosa, or hyperthyroidism
   b. Ovarian hyperfunction is usually caused by estrogen-secreting tumors or may be idiopathic in origin.
   (1) Primary ovarian hyperfunction is the result of the presence of an estrogen-secreting tumor, which results in decreased serum FSH/LH levels. Irregular uterine bleeding is a typical symptom.
   (2) Secondary ovarian hyperfunction is idiopathic and results in increased estrogen secretion caused by the presence of increased FSH/LH levels. Sexual precocity (early development of secondary sex characteristics) is the primary condition associated with this disorder.

6. Other conditions involving female reproductive hormones include:
   a. Hirsutism, or excess hair along the midline of the female body (i.e., lip, chin, chest), is typically caused by excess androgen production by the ovaries or adrenal glands.
   b. Polycystic ovary syndrome, or enlarged ovaries, is associated with infertility and other menstrual irregularities.
   c. Infertility is caused by lack of ovulation in women and inappropriate sperm production in men.

7. Laboratory analysis of reproductive endocrinology involves serum and urine testing of a variety of hormones and their metabolites or function tests that involve stimulation or suppression of various hormones.

VII. TOXIC AND THERAPEUTIC DRUGS

A. Toxicology is the study of toxic drugs or poisons. A toxicant (poison) is any substance that, when taken in sufficient quantity, causes sickness or death. Toxicity is a relative term used to compare one substance with another; a toxic substance is one with a toxicity defined as “extremely” or “super” toxic.

B. Specific drugs considered to be toxicants are composed of several categories. Some of these drugs are also considered to be therapeutic in nature.
1. Analgesics are anti-inflammatory agents and painkillers. These drugs are also considered to be therapeutic.
   a. Salicylate (aspirin) is considered toxic at a serum level of >90 mg/dL 6 hours following ingestion. The time since ingestion must be known to determine severity of toxicity.
      (1) Salicylate intoxication results in the following: stimulation of the respiratory system with initial respiratory alkalosis, conversion of pyruvate to lactate, inhibition of oxidative phosphorylation, and breakdown of fatty acids to produce ketoacids. Eventually, metabolic acidosis occurs.
      (2) Renal clearance of salicylate can be increased by forced alkaline diuresis.
   b. Acetaminophen, if present in serum at 300 mcg/mL 2 hours after ingestion, induces hepatic toxicity. Again, time since ingestion is critical in determining acetaminophen intoxication.
      (1) Intoxication results in hepatocystic necrosis 3 to 4 days after overdose because of the inability of the liver to adequately conjugate the metabolite of acetaminophen (i.e., acetamidoquinone) to glutathione. High levels of acetamidoquinone in the liver induce hepatocyte death.
      (2) Antidote. The effective antidote for acetaminophen overdose is N-acetylcysteine, which is thought to act as a glutathione substitute and binds to the metabolite.

2. Barbiturates are short-acting (pento-, secobarbital); intermediate-acting (amo-, β-, butabarbital); and long-acting (phenobarbital) sedatives that exert a tranquilizing effect through their depressant effect on the CNS. The barbiturates can be considered as substances of abuse or as analgesics.
   a. Barbiturate intoxication results in cardiac arrest and respiratory depression through its effect on the CNS.
   b. There are no real antidotes for barbiturate overdose except the establishment of an open airway, aiding respiration, and maintaining cardiac output.

3. Narcotics (opioids) are compounds include heroin, morphine, codeine, and methadone. Most of these drugs are habit forming and can be considered drugs of abuse.
   a. Intoxication. The toxic effects of overdose include depression of respiration caused by a decreased sensitivity to carbon dioxide and coma. Heroin is metabolized by the liver to form morphine and is excreted by the kidney as morphine glucuronide.
   b. Antidote. The effective antidote for narcotic overdose is naloxone, a narcotic antagonist.

4. Pesticides exist as organic complexes, organophosphates (largest single group of pesticides), and carbamates. These compounds inhibit AChE, which results in specific effects on the heart and respiratory centers, muscle cramps, and certain CNS effects.
   a. Intoxication. The method used for the determination of pesticide poisoning is the examination of PChE (an isoenzyme of AChE found in serum).
   b. Antidote. The effective antidote for pesticide poisoning is atropine sulfate.

5. Carbon monoxide is a tasteless gas with 200-fold greater affinity for hemoglobin than oxygen.
   a. Intoxication. During carbon monoxide poisoning, hemoglobin cannot adequately exchange carbon dioxide for oxygen because of the increased amount of carboxyhemoglobin present. Suffocation and death follow.
   b. Antidote. The effective antidote for carbon monoxide poisoning is removal of the source of carbon monoxide or removal of the victim from the source.

6. Metal poisoning includes intoxication by the heavy metals lead, arsenic, and mercury.
   a. Lead poisoning is most typically caused by lead paint ingestion or continuous exposure to lead in the soil.
      (1) Intoxication. Lead is found primarily in RBCs in intoxicated victims but produces widespread effects, such as gastrointestinal irritation, weight loss, kidney damage, convulsions, and in children, altered cognition and encephalopathy. Death occurs because of peripheral vascular collapse or brain involvement.
      (2) Antidote. Administration of ethylenediaminetetraacetic acid (EDTA), penicillamine, and other lead chelates that bind the lead and allow it to be excreted by
b. **Mercury (mercury salts)** is found in antibacterial agents, photographic reagents, pesticides, and batteries. Poisoning by these agents is typically the result of over-exposure or ingestion.

   1. **Intoxication.** Effects of mercury poisoning include gastrointestinal irritation, severe kidney damage, or neurologic symptoms.
   2. **Antidote.** As with lead poisoning, chelates, such as EDTA, penicillamine, or dimercaprol (also referred to as British antilewisite (BAL)) bind the mercury and render it able to be excreted.

c. **Arsenic** is found in pesticides, weed killer, and some paints.

   1. **Intoxication.** Arsenic induces purging gastroenteritis, shredding of the stomach lining, and causes Mees’ lines in the fingernails because of keratin binding. Death typically occurs because of hemorrhagic gastroenteritis. Because arsenic is cleared rapidly from the blood, urine is the specimen of choice for analysis.
   2. **Antidote.** Chelation therapy with penicillamine or BAL is effective.

7. **Substances of abuse** include a variety of compounds that, when found in high levels in the urine or serum, can incriminate an individual. It is mandatory in substance-of-abuse analysis to obtain a satisfactory specimen and to process the specimen in a secure part of the laboratory.

a. **Ethanol** is the most common toxicant and substance of abuse seen in patients in emergency departments in the United States. It acts by depressing the CNS and increasing the heart rate and blood pressure. Ethanol **metabolism** occurs in the liver by alcohol dehydrogenase, which converts alcohol to acetaldehyde, which is then excreted to acetate. A blood level of 0.1 g/dL is defined as intoxicating.

b. **Methanol** is widely used in paints, solvents, antifreeze, and solid canned fuels. Poisoning with methanol is typically caused by ingestion.

   1. **Intoxication** by methanol is considered more dangerous than ethanol poisoning because of the formation in humans of formaldehyde and formic acid as metabolites. Formic acid formation results in metabolic acidosis, pancreatic necrosis, and visual system impairment.
   2. **Antidotes** for methanol poisoning include the administration of sodium bicarbonate to treat the acidosis and ethanol to bind alcohol dehydrogenase and block the formation of the metabolites.

c. **Amphetamines** are CNS stimulants that block dopamine receptors in the brain. Amphetamine **metabolism** occurs in the liver and produces benzoic acid.

   1. **Intoxication** by amphetamines can produce severe depression, respiratory difficulties, and episodes of paranoia.
   2. **Antidote.** The antidote for amphetamine overdose is forced acid diuresis.

d. **Cocaine** is a CNS stimulant that is metabolized by cholinesterase to form benzoylecgonine, which is excreted by the kidney.

   1. **Intoxication.** Cocaine overdose produces hypertension, myocardial infarction, or seizure. Cardiotoxicity can occur and result in sudden death following cocaine use.
   2. **Antidote.** There is no antidote for cocaine overdose except the passage of time and urinary excretion.

e. **Cannabinoid compounds** (tetrahydrocannabinol (THC), marijuana) produce psychologic effects and are stored in fat cells. THC is excreted in the urine for an extended period of time depending on use. Overdose of this drug is rare and is not severe enough to be life-threatening.

f. **Phencyclidine (PCP, angel dust)** is an abused anesthetic that is illegally used as a hallucinogen.

   1. **Intoxication.** This drug can produce violence, seizures, respiratory depression, or death.
   2. **Antidote.** Treatment for PCP overdose is diazepam. PCP is unmetabolized and excreted in the urine as phencyclidine.
C. The drug screen rapidly identifies a drug or drugs present in the blood, urine, or gastric contents of a patient suffering from toxicity. Neutral and basic drugs as well as drug metabolites are best detected in urine, whereas acidic drugs are best found in detectable concentrations in blood and serum. Following a positive drug screen, confirmatory methods must be used to quantitatively analyze drug levels in a patient.

1. Handheld immunoassays are the most common type of drug screen. They detect a wide variety of drugs but cannot separate closely related compounds. Blood and urine can both be analyzed with this method.

2. Gas-liquid chromatography allows for greater sensitivity in the identification of drugs. It can be used as a confirmatory technique for drugs detected by drug screen.

D. Confirmatory drug tests are required to confirm and quantify those drugs found in a patient’s serum or urine using drug-screening methods.

1. Gas chromatography/mass spectrometry is a sensitive technique used to confirm drugs detected by screening techniques. Typically, urine samples are initially analyzed by gas chromatography to determine the presence of compounds, then reanalyzed by mass spectrometry to examine fragments of these compounds for relative abundance in the sample.

2. Immunoassay techniques use antibodies to detect drugs. These methods are usually automated and in the form of enzyme immunoassays.

3. Ethanol testing is typically performed using gas chromatography. However, an enzyme assay using alcohol dehydrogenase and measuring the increase in NADH formation following the reaction is widely used and can be automated.

4. Heavy metal testing is most often performed by atomic absorption spectrophotometry.

E. Therapeutic drugs must be monitored to determine what doses are inadequate or excessive in the treatment of the patient. Often, the ingested drug (called the “parent” drug) is metabolized to form an active metabolite that produces an effect similar to the parent drug.

1. Cardiac drugs are divided into two categories: the cardiac glycosides and the antiarrhythmic drugs. These agents serve to maintain normal heart function.

a. Digoxin is the major cardiac glycoside and alters the force of contraction through its effect on the ATPase pump in heart muscle. Blood specimens must be collected 8 hours after a dose of digoxin is administered, because its peak concentration in tissue occurs 6 to 10 hours after administration. Digoxin toxicity produces symptoms of nausea, rapid heart rate, and visual impairment. Digoxin is excreted as digoxigenin in the urine.

b. The antiarrhythmic drugs are prescribed to treat irregular heartbeat that produces inappropriate ventricular contraction or tachycardia (increased heart rate).

(1) Lidocaine is used for the treatment of faulty ventricular contractions and arrhythmias. It binds to α1-acid glycoprotein and is metabolized in the liver, producing two active metabolites, monomethylglycinexylidide and glycineexylide.

(2) Procainamide is used to treat inappropriate ventricular contractions and tachycardia. This drug is metabolized in the liver to form an active metabolite, N-acetylpriorcainamide, which produces the same effect as its parent drug. Therefore, serum levels of both drugs must be analyzed.

(3) Disopyramide stabilizes the heartbeat. It is both excreted by the renal system as the unchanged drug and is metabolized in the liver to form an inactive metabolite.

(4) Quinidine is a myocardial depressant that decreases the heart’s ability to conduct current. It is metabolized in the liver to produce several active metabolites, including 3-hydroxyquinidine. If quinidine is added to a digoxin therapy regimen, an interaction occurs that induces an increase in digoxin concentration.

(5) Propranolol is prescribed for atrial and ventricular arrhythmias and hypertension. It is considered to be a beta-blocker.

2. Anticonvulsants function to alter transmission of nerve impulses within the brain to minimize the seizures of epilepsy.
a. Phenobarbital is used to treat all types of seizures except absence seizures. It is effective in children and neonates. It is metabolized in the liver, and serum concentrations increase during the administration of valproic acid or salicylic acid.

b. Phenytoin corrects grand mal seizures. It is metabolized by the liver and can interact with several drugs that induce increased serum concentration or increased metabolism of phenytoin.

c. Valproic acid is prescribed for absence (petit mal) seizures. Valproic acid affects many others anticonvulsants by inhibiting their metabolism in the liver, thus increasing serum concentration.

d. Primidone is metabolized in the liver to form phenobarbital. Therefore, dual analyses must be performed to determine the proper dosage of this drug. It is used to treat both grand mal and complex-partial seizures.

e. Carbamazepine is typically used for treatment of various seizures and facial pain.

f. Ethosuximide is prescribed for the treatment of petit mal seizures.

3. Bronchodilators act to relax bronchial smooth muscle for relief or prevention of asthma. Theophylline is the most common in this category of therapeutic drugs and is metabolized in the liver to produce several metabolites, including caffeine.

4. Psychotropic or antipsychotic drugs are used to treat psychotic patients. They can be categorized in two classes: lithium and the antidepressants.

a. Lithium treats manic-depressive illness. The mechanism of action of lithium as a mood stabilizer remains unknown, although effects on synaptic neurotransmission are thought to be the cause. Lithium is filtered by the renal glomerulus and eliminated as the unchanged drug.

b. Antidepressants, or tricyclic antidepressants, are used to treat depression that has no apparent organic or social cause. Antidepressants include imipramine, nortriptyline, amitriptyline, and desipramine, all of which are metabolized by the liver to form active metabolites. The active metabolites include desipramine (parent is imipramine), nortriptyline (parent is amitriptyline), and 2-hydroxy-desipramine (parent is desipramine).

c. Fluoxetine is not chemically related to the tricyclic antidepressants, but has a similar effect by blocking serotonin uptake by nerve terminals in the CNS and by platelets.

5. Antineoplastic drugs are used in the management of certain tumors, including those found in breast, testicular, pharyngeal, and sometimes lung cancer. These agents work by inhibiting DNA synthesis.

F. Therapeutic drug monitoring (TDM) is performed to determine patient compliance to the drug-taking regimen, to monitor drug interactions, and to monitor drugs that are used for a preventive effect.

1. Changes in drug concentrations in the body, which occur with time, are related to the course of the pharmacologic effects. The change in drug concentration over time is described by the following steps.

a. Liberation is the release of this ingredient, followed by the process of the drug passing into solution.

b. Absorption is the process by which the drug molecule is taken up into systemic circulation. Following absorption through the intestinal mucosa, a drug traverses the hepatic system, where some drugs undergo substantial metabolism and elimination. This is called first-pass elimination or metabolism.

c. Drug molecules can be confined to the blood, leave the bloodstream, and enter the extravascular space, or they can migrate into various tissues. This is referred to as distribution, a process that typically occurs between a period of 30 minutes and 2 hours. The bioavailability of a drug is the amount of drug that is absorbed into the system and is available for distribution.

d. Metabolism is the process of transformation of the parent drug molecule to its metabolite(s). Metabolites are usually water soluble and can be easily excreted. Most of the metabolism occurs in the liver, where enzymes catalyze oxidation, reduction, or hydrolysis of the drug.
Elimination is the process of excretion of the drug from the body. Drugs are typically excreted in the urine but also can be eliminated in the feces, sweat, expired air, and saliva.

2. Basic principles. TDM measures drug concentrations during therapy with pharmaceutical agents.
   a. A steady-state drug level (complete with peaks and troughs) exists for each drug. When a single dose of a drug is administered orally, the blood level changes markedly over time and, at some time, the concentration in the plasma reaches its peak (highest point) and then declines. Immediately before the next dose of medication, a trough level occurs.
      (1) For single-dose administration, the rate of decline in concentration is expressed in terms of half-life, which is the time required for the concentration of the drug to decrease by 50% (Figure 1–6). The half-life is different for each drug.
      (2) At steady-state levels, the rate of administration of the drug is equal to the rates of metabolism and excretion, allowing the drug level to remain constant.

G. Pharmacokinetics is the mathematical interpretation of drug disposition over time to determine proper dosing amounts of a therapeutic drug. Pharmacokinetic responses are typically graphic plots of blood concentration of the drug versus time, such as a dose-response curve (see Figure 1–6). Three kinetic processes are used to describe the fate of drugs in the body over a period of time and can be illustrated in a dose-response curve.
   1. First-order kinetics describe absorption, distribution, and elimination of drugs. This means that the rate of change of concentration of a drug is dependent on the drug concentration. It is represented by the first phase of the dose-response curve.
   2. Zero-order kinetics describe the rate of change of concentration of a drug that is independent of the concentration of the drug. That is, a constant amount of drug is eliminated per unit of time. This typically depends on the ability of the liver to metabolize the drug. This is illustrated by the second phase of the curve.
   3. Michaelis-Menten kinetics state that if a drug concentration in a system exceeds the capacity of the system, the rate of change of concentration proceeds according to the Michaelis-Menten equation.

H. Laboratory analysis of therapeutic drugs includes enzyme immunoassays and fluorescence-polarized immunoassays. Gas chromatography and high-pressure liquid chromatography are also used, particularly as confirmatory tests when a screening test is positive. Serum is typically the specimen of choice for drug analysis, but urine metabolites are measured in some cases, particularly in screening tests.

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CHAPTER 2
Hemostasis and Coagulation

JOEL HUBBARD, PhD, MT (ASCP)

I. PLATELET PHYSIOLOGY

A. General considerations. Platelets, which exist in whole blood in concentrations of 150,000 to 440,000/mm³, are disk-shaped cells necessary for hemostasis. Platelets are formed from the cytoplasm of megakaryocyte in the marrow.

1. A Wright’s-stained blood film provides an estimation of platelet numbers, size, and distribution.

2. Platelet number can be obtained by manual platelet count (hemacytometer) or an electronic cell counter.

B. Platelet ultrastructure (Figure 2–1)

1. Glycocalyx is the outer membrane surface. It is rich in glycoproteins, which serve as membrane receptors.
   a. Glycoprotein Ib is the receptor for von Willebrand’s factor (vWF) in the presence of ristocetin.
   b. Glycoproteins IIb and IIIa are receptors for vWF and fibrinogen and are exposed by stimulation of thrombin or adenosine diphosphate (ADP).
   c. Glycoprotein Va is the receptor for thrombin.

2. Microtubule and micro filaments. These provide an active means of platelet contraction to squeeze out the contents of the cytoplasmic granules.
   a. Microtubules form the submembranous band around the circumference of the cell and structurally support the normal discoid-shaped platelets.
   b. The contractile microfilaments (thrombozoethin) contain actin and are closely related to the microtubule.

3. The open canalicular system provides direct communication between intracellular and extracellular compartments.

4. A dense tubular system forms a circle within the microtubule.
   a. This system serves as a site for arachidonic acid metabolism.
   b. This system also functions as a calcium-sequestering pump that maintains platelet cytoplasmic calcium levels.

5. Mitochondria are responsible for energy production.

6. Glycogen granules provide energy substrate.

7. Alpha (α) granules contain contact-promoting factors, including:
   a. Platelet fibrinogen
   b. Platelet-derived growth factor (PDGF)
c. von Willebrand’s factor (factor VIII:R)
d. β-Thromboglobulin (BTG)
e. Platelet factor 4 (heparin-neutralizing)
f. Fibronectin

8. Dense granules contain nonprotein factors including:
   a. Adenosine diphosphate (ADP)
   b. Adenosine triphosphate (ATP)
   c. 5-Hydroxytryptamine (5-HT; or serotonin)
   d. Calcium

C. Platelet function

1. Damaged subendothelium releases factors that activate the platelet, transform its shape, and evolve a “sticky” platelet aggregate to plug the leak. These factors include:
   a. Collagen
   b. Fibronectin
c. vWF (factor VIII:R)
d. Thrombin
e. ADP

2. Tissue platelet activators cause the platelet to change shape from discoid to spherical. Dense and α-granules undergo internal contraction and centralization. The complete process is calcium dependent.
   a. The exposure of surface membrane receptors to vWF and fibrinogen results in cytoplasmic calcium ionization, stimulation of ATP generation, and activation of the actin monomers in the microfilaments.
   b. Contractions result in a centralization of the cytoplasmic granules and a release of their contents through the canalicular system.

3. Plug formation, or secondary aggregation, is primarily stimulated by thrombin and thromboxane A₂ (TXA₂).
   a. Membrane-binding of vWF and collagen to platelet receptors unmask membrane phospholipid A₂, which is the precursor of arachidonic acid, and is important for the production of TXA₂ and other prostaglandins. Phospholipid A₂ is unmasked by the binding of vWF and collagen to platelet receptors on the membrane.
   b. TXA₂ inhibits adenylate cyclase [thus, it inhibits the formation of cyclic adenosine monophosphate (cAMP)] and liberates sequestered calcium into cytoplasm. Calcium causes further cytoplasmic contraction, release of granule contents, and platelet aggregation.
   c. Thrombin enzymatically cleaves fibrinogen to form fibrin, which is necessary to stabilize the platelet plug.
4. Growth-limiting factors of the platelet aggregate include:
   a. Blood flow, which washes away coagulation-promoting factors
   b. Release of prostaglandin PGI₂ (prostacyclin) by the surrounding vascular tissues

5. Granular release of substances from dense granules, such as serotonin, prostaglandins (except for TXA₂), and lysozymes, causes local inflammation and vasodilation, which increases blood flow.

D. Kinetics

1. Megakaryocytopoiesis development occurs by endomitosis (i.e., nuclear splitting without cell division).
   a. A single megakaryoblast nucleus may contain 2 to 64 times the normal number of chromosomes.
   b. Nuclear chromatin is densely staining, dispersed early, and more compact at later stages.
   c. Nucleoli are small at all development stages.

2. Stages of megakaryocytic maturation (Figure 2–2)
   a. Megakaryoblasts descend from a unipotential stem cell (CFU-Meg). They are characterized by overlapping nuclear lobes and basophilic staining cytoplasm; their size ranges from 6 to 24 μm.
   b. Promegakaryocytes are larger than megakaryoblasts (14 to 30 μm in diameter) and have more cytoplasm.
      (1) The nucleus becomes increasingly lobulated and spreads out into a horseshoe shape.
      (2) Red-pink granules are visible in the center of the cell.
   c. Granular megakaryocytes (16 to 56 μm) are characterized by increased spreading of nuclear lobes and spreading of pink granules throughout the cytoplasm.
   d. Mature megakaryocytes (20 to 50 μm) have a compact nucleus, and the basophilia of cytoplasm has disappeared.
      (1) Platelet fields are clusters of pink granules in the cytoplasm.
         (a) Platelet fields are produced by an invagination of surface membrane, separating the cytoplasm into individual platelets.

<table>
<thead>
<tr>
<th>Megakaryoblast</th>
<th>Promegakaryocyte</th>
<th>Granular megakaryocyte</th>
<th>Mature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Size range</td>
<td>0 – 24 μm</td>
<td>14 – 30 μm</td>
<td>15 – 56 μm</td>
</tr>
<tr>
<td>Cytoplasmic staining</td>
<td>Deep blue (basophilic)</td>
<td>Basophilic with pink center</td>
<td>Mostly pink</td>
</tr>
<tr>
<td>Granules</td>
<td>Rare</td>
<td>Few</td>
<td>Extensive</td>
</tr>
<tr>
<td>Nuclear morphology</td>
<td>Few compacted lobes</td>
<td>Lobes spread out in horseshoe shape</td>
<td>Many lobes spread out</td>
</tr>
<tr>
<td>N:C ratio</td>
<td>High</td>
<td>Moderate</td>
<td>Moderate</td>
</tr>
</tbody>
</table>

Figure 2–2 Morphologic characteristics of stages of megakaryocytic maturation comparing the nuclear and cytoplasmic variations of each stage. N:C, nuclear-cytoplasmic ratio.
Individual platelets are shed from the megakaryocyte cytoplasm into the marrow sinuses, and then are released into the vascular lumen.

Each mature megakaryocyte produces from 2,000 to 7,000 platelets that each range in size from 2 to 3 μm.

Maturation time from the blast stage to platelet formation is typically 5 days.

Normal marrow contains approximately 15 million megakaryocytes. This equates to approximately 5 to 10 megakaryocytes per 10 x power field when bone marrow smears are microscopically examined.

Normal circulation life of a platelet is 8 to 10 days.

Platelets are removed by macrophage in the liver and spleen or by active use in daily coagulation mechanisms.

Circulating platelets are distributed between the spleen and blood.

- One third of the circulating platelets are always in the spleen.
- The platelet count is higher in patients without a spleen and lower in patients with splenomegaly (enlarged spleen).

Regulation of the platelet count

- Under normal conditions the platelet count (or mass) is constant, even with active use. This indicates a feedback system that adjusts production to consumption.
- Rebound thrombocytopenia occurs after platelet transfusion.
- Rebound thrombocytosis occurs after platelet depletion.

Feedback stimulus results in an increased megakaryoblast endomitosis, which increases platelet volume and number. It also affects committed unipotential stem cells, which results in more megakaryoblasts.

Laboratory measurements of platelet activities

1. Initial evaluation includes a platelet count and slide estimate, with a reference value of 150,000 to 440,000/mm³.

2. Bleeding time is an effective in vivo screening test of platelet function by timing the length it takes for platelets to plug broken capillaries after a small cut is made in the forearm.
   - Reference values are approximately 3 to 8 minutes.
   - Increased bleeding times are seen in:
     - Patients taking drugs with antiplatelet action (e.g., aspirin)
     - Patients with von Willebrand’s disease (vWD)
     - Patients who suffer from congenital platelet abnormalities
     - Patients with platelet counts lower than 100,000/mm³

3. Platelet aggregation is measured with a platelet aggregometer.
   - Basic principle: Citrated, platelet-rich plasma is stirred in the aggregometer while a light beam is passed through the suspension.
   - A chemical stimulus is added (e.g., collagen, epinephrine, ADP, ristocetin, A23187 (calcium ionophore), arachidonic acid, γ-thrombin (partially trypsinized thrombin)).
   - γ-Thrombin retains aggregating properties but lacks clotting ability.
   - The shape change from discoid to spheroid is monitored as an initial decrease in light transmittance.
   - Subsequent aggregation allows an increase of light to pass through the suspension to the photodetector and to be recorded as an increase in light transmittance.

II. PLATELET PATHOPHYSIOLOGY

A. Quantitative platelet disorders (Figure 2–3)

1. Thrombocytopenia is characterized by a decrease in the number of circulating platelets (i.e., <100,000/mm³). Clinical evidence of thrombocytopenia includes an increased number of petechiae, hemorrhages, prolonged bleeding time, and impaired clot retraction. Decreased circulating platelet counts result from the following conditions:
   - Low platelet counts can result from defective production in the bone marrow.
Figure 2-3: Platelet disorder algorithm. DIC, disseminated intravascular coagulation; IDA, iron-deficiency anemia; ITP, idiopathic thrombocytopenic purpura; TTP, thrombotic thrombocytopenic purpura; TXA$_2$, thromboxane A$_2$; SLE, systemic lupus erythematosus.

1. Decreased numbers of megakaryocyte are seen in the following disorders:
   (a) Congenital disorders (e.g., Fanconi’s anemia, maternal infection)
   (b) Acquired disorders seen with the use of radiation, alcohol, thiazide diuretics, chloramphenicol, and cancer chemotherapy
   (c) Marrow replacement by malignant cells, which occurs with metastatic carcinoma, leukemia, lymphoma, myeloma, and myelofibrosis

2. Ineffective platelet production in the marrow is seen with all of the following conditions:
   (a) Hereditary thrombocytopenia
   (b) Vitamin B$_12$ or folate deficiency (megaloblastic anemia)
   (c) Di Guglielmo’s syndrome (also known as erythroblastosis)
   (d) Proximal nocturnal hemoglobinuria (PNH)

b. Thrombocytopenia can result from disorders of distribution and dilution of platelets in the circulation. These conditions include:
   (1) Splenic pooling, which is commonly seen with splenomegaly and hypersplenism
   (2) Hypothermia, which results in vascular shunting
   (3) Dilution in the circulation by transfused stored blood

c. Thrombocytopenia can result from disorders that result in the destruction of platelets.
   (1) Combined consumption of both platelet and coagulation factors is seen with:
      (a) Toxicity due to snake venoms
      (b) Tissue injury
      (c) Obstetric complications (e.g., aborted fetus, toxemia of pregnancy)
      (d) Neoplasms (e.g., promyelocytic leukemia, carcinoma)
      (e) Bacterial and viral infections
      (f) Intravascular hemolysis
(2) Isolated consumption of platelets results from the following disorders:

(a) Thrombotic thrombocytopenia purpura (TTP) is caused by excessive deposition of platelet aggregates in renal and cerebral vessels. Physiologically, TTP is believed to be caused by vascular wall dysfunction, which disrupts the inert basement membrane.

(i) Clinical diagnosis. TTP is three times more prevalent in women than men at an average age of 35 years. The majority of patients are seen with microangiopathic hemolytic anemia, thrombocytopenia, neurologic symptoms (e.g., headaches, seizures), fever, and renal disease.

(ii) Laboratory diagnosis of TTP includes a prothrombin time (PT) that is normal in 88% of patients, an activated partial thromboplastin time (APTT) that is normal in 94% of patients, and a fibrinogen level that is normal in 79% of patients. Fibrin-degradation products (FDP) are normal in 53% of patients, weakly positive in 23%, and positive in 24%.

(iii) Pathophysiology of TTP possibly results from vascular endothelial cell damage, an increase in a platelet-aggregating factor, a possible deficiency of platelet-aggregating factor inhibitor, a decrease in prostacyclin (PGI2), an increase in PGI2 degradation, or an absence of a plasminogen activator.

(b) Hemolytic uremic syndrome

c) Vasculitis [as seen with systemic lupus erythematosus (SLE)]

d) Disseminated intravascular coagulation (DIC)

(3) Immune destruction of platelets occurs in the following disorders:

(a) Idiopathic (immunologic) thrombocytopenia purpura (ITP) is an autoimmune disorder (Table 2–1). Common laboratory findings include an increase in mean platelet volume (MPV), decreased platelet count, increased bone marrow platelet production, increased marrow megakaryocyte, a normal bleeding time, and platelet-associated IgG.

(b) Acute ITP occurs in children 2 to 6 years of age. There is a sudden onset of thrombocytopenia, which often follows viral infections, such as rubella, chickenpox, cytomegalovirus (CMV), and toxoplasmosis.

(i) Duration. Acute ITP usually lasts for 2 to 6 weeks with a spontaneous remission in 80% of patients.

(ii) Platelet count is usually <20,000/mm³ in patients with acute ITP.

(iii) Pathophysiology. Acute ITP is caused by viral attachment and antigenic alteration of platelet membrane proteins that result in the formation of platelet autoantibodies, which are most often IgG. IgG-coated platelets are removed by macrophages in the spleen.

(iv) Therapy. Because acute ITP is usually self-limited, corticosteroids are the treatment of choice when therapy is instituted. Steroids

<table>
<thead>
<tr>
<th>Table 2–1</th>
<th>Acute and Chronic Idiopathic Thrombocytopenia Purpura: A Comparison</th>
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<tbody>
<tr>
<td></td>
<td>Acute Idiopathic Thrombocytopenia Purpura</td>
</tr>
<tr>
<td>Onset</td>
<td>Children aged 2–6 y; sudden onset, often after viral infections</td>
</tr>
<tr>
<td>Duration</td>
<td>2–6 wk; spontaneous remission in 80%</td>
</tr>
<tr>
<td>Platelet count</td>
<td>&lt;20,000/mm³</td>
</tr>
<tr>
<td>Pathophysiology</td>
<td>Altered platelet-membrane proteins cause formation of platelet autoantibodies</td>
</tr>
<tr>
<td>Therapy</td>
<td>Corticosteroids</td>
</tr>
</tbody>
</table>
suppress macrophage phagocytic activity, decrease Fc-receptor function, and decrease antibody platelet binding. Splenectomy is rarely needed, and platelet transfusion is ineffective.

(e) Chronic ITP occurs in adults 20 to 40 years of age. It is found in women three times more than in men and has a slow, asymptomatic onset of thrombocytopenia.

(i) Duration. Chronic ITP can last from months to years.

(ii) Platelet count usually ranges from 30,000 to 80,000/mm³.

(iii) Pathophysiology. Chronic ITP is often associated with SLE.

(iv) Therapy. Splenectomy is the most common treatment because it decreases the number of macrophage with Fc receptors. Immuno-suppressive chemotherapy with vincristine or vinblastine is used in severely affected patients.

(d) Posttransfusion purpura occurs in 1% to 2% of persons who receive blood transfusions. Production of antiplatelet antibodies by the recipient of platelet transfusions results in the destruction of platelets.

(e) Isoimmune neonatal purpura is caused by maternal viremia (e.g., CMV or rubella) or maternal drug ingestion.

(f) Drug-induced antibody formation is most commonly seen with the use of quinidine and heparin. The drugs function as haptens, combining with a serum protein and causing an antibody response. The drug-antibody complex attaches to platelets, which results in agglutination, complement fixation, and destruction by macrophages.

d. Thrombocytopenia can result from a combination of mechanisms (e.g., alcoholism, lymphoproliferative disorders).

e. Heparin-induced thrombocytopenia is observed in more than 10% of patients who undergo heparin therapy.

(1) Risk. From thrombosis without heparin therapy is greater than the risk of bleeding from heparin-induced thrombocytopenia.

(2) The mechanism of thrombocytopenia is due to a direct platelet-aggregating effect, as well as immune destruction by antiplatelet antibodies.

(3) Laboratory diagnosis. A normal aggregation pattern in platelet aggregometer studies is found, except that adding heparin as a stimulant will increase aggregation instead of blunting the aggregation reaction.

f. Thrombocytopenia associated with human immunodeficiency virus (HIV) infection is severe but rarely hemorrhagic.

(1) Characteristics similar to classic ITP include:

(a) Abundant megakaryocytes
(b) Occasional giant platelets
(e) Immune origin
(d) Absence of splenomegaly

(2) Characteristics different from ITP include:

(a) Greater levels of bound antibody
(b) Involvement of immune complexes

2. Thrombocytosis is characterized by an increase in circulating platelet counts >450,000/mm³.

a. Essential thrombocytosis is the result of a primary bone marrow disorder. Although it is characterized by an increased number of platelets, it is caused by a clonal proliferation that affects all hemopoietic cells. Often, patients with a thrombocytosis will have increased bleeding tendencies because of possible accompanying functional abnormalities. Essential thrombocytosis is most commonly seen in patients with the following disorders.

(1) Hodgkin’s lymphoma
(2) Polycythemia vera
(3) Myelofibrosis
(4) Chronic myelogenous leukemia
(5) Thrombocytemia
b. Secondary thrombocytosis is a secondary response most commonly associated with the following disorders:

1. Iron-deficiency anemia associated with chronic blood loss
2. Chronic inflammatory disease may be associated with high platelet counts
3. Splenectomy-associated thrombocytosis
4. Rebound thrombocytosis, which may occur after a platelet depletion through a massive blood loss

B. Qualitative platelet abnormalities

1. Surface membrane defects that are genetically acquired
   a. Glanzmann thrombasthenia
      (1) This disease is a homozygous autosomal recessive disorder in which one of two genes coding for either the membrane receptor glycoprotein IIb (GPIIb) or GPIIIa is affected. Both genes are found on chromosome 17. Males as well as females are affected.
      (2) Both receptors function in the aggregation process to anchor platelets to exposed collagen in the subendothelial tissue of damaged blood vessel walls. The blood clot fails to form, because the dimer complex is no longer defective and platelets can no longer bind to one another.
      (3) Symptoms commonly present in infants and in early childhood.
         (a) Bruising (i.e., purpura)
         (b) Nose bleeds
         (c) Bleeding from the gums started by trauma or simple sneezing, crying, coughing, or the eruption of a tooth
         (d) Female adolescents may experience heavy menorrhagia when they begin their cycle.
         (e) In many, the severity of bleeding events lessens with age.
      (4) Laboratory diagnosis of this disorder is for the most part straightforward.
         (a) Normal platelet count and morphology
         (b) Significantly prolonged bleeding time
         (c) Platelet aggregometer profile shows failure to aggregate with all agents (i.e., ADP, adrenaline, thrombin, collagen), except von Willebrand factor.
      (5) Clinical variants of Glanzmann’s have been identified based on their patterns of severity.
         (a) Type I is a severe deficiency of <5% of the glycoprotein complex.
         (b) Type II is considered a mild to moderate deficiency with 5% to 20% of the complex remaining.
         (c) Clinical Type III is found to be a qualitative dysfunction.
      (6) Prognosis and treatment is of a lifelong disorder. Glanzmann’s now is associated with a low death rate from hemorrhage with modern medical care and support.
         (a) The only curative treatment for Glanzmann’s disorder is the bone marrow transplant.
         (b) Most treatments are supportive of the bleeding and preventive management.
         (c) Treatment of bleeding episodes can include platelet transfusion or local application of thrombin and adrenaline solutions.
   b. Bernard-Soulier syndrome (BSS)
      (1) Is directly due to an inherited quantitative or qualitative defect in the platelet glycoprotein (GP) Ib/IX complex consisting of four platelet surface glycoproteins (Ib\(^{\alpha}\), Ib\(^{\beta}\), IX, and V),
         (a) The GP Ib\(^{\alpha}\) gene is located on the short arm of chromosome 17, the GP Ib\(^{\beta}\) gene is on the long arm of chromosome 22, and the GP IX and GP V genes are located on the long arm of chromosome 3.
         (b) The Ib molecule carries both the high- and moderate-affinity receptor sites for thrombin.
(c) This receptor complex mediates adhesion of platelets to the blood vessel wall at sites of injury by binding vWF and facilitates the ability of thrombin to activate platelets.

(d) BSS is extremely rare, occurring <1 per 1 million.

(2) Clinical symptoms can present early in life and most commonly include frequent bouts of epistaxis, gingival and cutaneous bleeding, and hemorrhage associated with trauma.

(3) Laboratory diagnosis commonly results in a prolonged Ivy bleeding time, giant platelets, and thrombocytopenia.

(a) Platelet counts may range from very low (i.e., <30,000/mm³) to slightly low or low normal.

(b) Bleeding time results range from slightly prolonged (8–10 minutes) to significantly prolonged (>20 minutes).

(c) Examination of a blood smear and the MPV for giant platelets.

(d) Platelet aggregation studies will show a reduced aggregation with ristocetin and a normal response to other agonists.

(4) Because BSS involves gene mutations of several glycoproteins codes by several different chromosomes, several clinical variants of the disorder have been identified.

(a) Type A clinical variant is what is known as classic BSS due to mutation in the GP Ibβ gene. A functional defect is often possible in which the GP Ibα is expressed in a dysfunctional form that fails to bind ligand.

(b) Type B variants have also been identified where the genetic lesion may be localized to the GP Ibα gene on chromosome 17, 22, or the GP IX gene on chromosome 3.

(c) Type C variants are due to GPV gene lesions on chromosome 3.

(d) Rarely, cases of acquired BSS have been described in patients with myelodysplasia and with acute myelogenous leukemia (M6).

(5) Prognosis and treatment of these patients concentrates on supportive measures and the treatment of acute bleeding episodes.

(a) Management should include the education of the patient and their families about their condition and avoiding unnecessary risks, antiplatelet medication such as aspirin or a nonsteroidal anti-inflammatory, and adequate dental hygiene.

(b) Iron-deficiency anemia often develops due to chronic bleeding.

(c) Splenectomy lessens the degree of thrombocytopenia and the severity of some symptoms.

(d) Often the transfusion of blood and platelets is necessary to control hemorrhage and thrombocytopenia with a traumatic event.

(e) In future modes of treatment, BSS may be an ideal candidate for gene therapy. Bone marrow or stem cell transplantation is still experimental for individuals with BSS.

c. Differential diagnosis of membrane-related disorders;

(1) Glanzmann’s thrombasthenia and BSS have many clinical and physiological similarities (Table 2–2).

(a) Both result in chronic bleeding of mucous membranes. Treatments and diagnosis will be different so it is important that a correct diagnosis is made.

(b) Both disorders will have an increased bleeding time, but the platelet count is commonly decreased in BSS with giant platelets.

(c) The aggregation patterns will typically give opposite results for each disorder.

(2) Bernard Soulier must be further differentiated from von Willebrand’s disease or ITP.

(a) Von Willebrand’s disease also presents with an increased bleeding time and impaired Ristocetin aggregation, but there is a decrease in vWF (VIII R), not in the receptor binding site.
Table 2–2  Differential Diagnosis of Glanzmann’s Thrombasthenia and Bernard-Soulier Syndrome

<table>
<thead>
<tr>
<th>Disorder</th>
<th>Bleeding Time</th>
<th>Platelet Count</th>
<th>MPV</th>
<th>Aggregation Pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glanzmann’s Thrombasthenia</td>
<td>Increased</td>
<td>Normal</td>
<td>Normal</td>
<td>Failure of normal aggregation pattern except Ristocetin</td>
</tr>
<tr>
<td>Bernard-Soulier Syndrome</td>
<td>Increased</td>
<td>Normal to decreased</td>
<td>Increased</td>
<td>Normal aggregation with all agents except Ristocetin</td>
</tr>
<tr>
<td>vWD</td>
<td>Increased</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal aggregation with all agents except Ristocetin</td>
</tr>
<tr>
<td>ITP</td>
<td>Normal to Increased</td>
<td>Decreased</td>
<td>Increased</td>
<td>Normal</td>
</tr>
</tbody>
</table>

(b) The addition of exogenous vWF in plasma cryoprecipitate to vWD patients will correct this bleeding disorder, but will not reverse the symptoms of those with BSS.

c) ITP also presents with a thrombocytopenia and giant platelets (i.e., MPV), but responds well to treatment with steroids and splenectomy, which is ineffective with BSS.

2. Abnormalities in the granular fraction of the platelet

a. Defects in dense granules. Some platelet functional abnormalities are due to abnormalities in the granular fraction of the platelet. Congenital deficiencies in dense granules, which contain ADP, ATP, serotonin, and calcium, show diminished platelet aggregation in the second wave of aggregation.

(1) Hermansky-Pudlak syndrome (HPS)

(a) Due to decreased numbers of platelet dense granules due to an autosomal recessive trait caused by mutations in the HPS1 gene on chromosome 10q23. The gene controls functions involved in the production and control of melanosomes, platelet dense bodies, and lysosomes resulting in a defect in platelet ADP release and a disruption in the ADP/ATP ratio granular content.

(b) Common symptoms consist of a triad phenotype of albinism, prolonged bleeding time, and the accumulation of ceroid pigment in lysosomal organelles.

(i) Other serious features include visual impairment, pulmonary fibrosis, inflammatory bowel disorder, and kidney disease.

(ii) The variant of albinism in HPS is a tyrosinase-positive form, with varied amounts of pigmentation. Some patients may have very light hair and frail features, whereas others may have dark hair and possess ocular albinism.

(iii) Organ dysfunction is due to accumulation of ceroid pigment in lysosomal organelles. Often the lungs are affected leading to pulmonary fibrosis due to remaining scar tissue.

(iv) Because of the platelet granular problems, easy bruising, frequent nose bleeds, prolonged wound bleeding, heavy menstrual bleeding in females, and excessive bleeding with dental procedures is common.

c) Increased bleeding time test may be the first clue to differential diagnosis.

(i) Laboratory diagnosis with standard blood tests, such as the PT, partial thromboplastin time (PTT), platelet count, and a bleeding time do not readily identify an individual with HPS.

(ii) Platelet aggregation studies may find a blunted response in the second phase of aggregation.
The most definite diagnosis can be found by electron microscope observation of the patient’s platelets and finding an absence of dense granules.

(2) Chediak-Higashi syndrome (CHS)
(a) CHS is a generalized autosomal recessive genetic disorder with recurrent infections in combination with ocular, neurological, and skin manifestations.
(i) Onset in early childhood and death often occurs before the age of 7.
(ii) Caused by mutations in the lysosomal trafficking regulator gene (LYST) found on chromosome 1.
(iii) Results in abnormal membrane fluidity, uncontrolled granule membrane fusion, and formation of giant cytoplasmic granules, and a lack of distinguishable dense granules in leukocytes, melanocytes, and platelets.
(b) The pathogenesis of CHS result in:
(i) Decreased (i.e., partial) pigmentation of the hair and eyes
(ii) Photophobia
(iii) Nystagmus
(iv) Abnormal susceptibility to infection
(v) Neutrophils demonstrate defective chemotaxis, degranulation, and bactericidal activity.
(vi) Platelets have decreased dense granule ADP resulting in an increased ATP:ADP ratio.
(vii) Neurological manifestations usually onset in adults with CHS and include motor, sensory, and cranial nerve defects.
(c) The laboratory diagnosis of CHS would include a CBC with platelet count, Wright’s stained blood smear, bleeding time, platelet aggregation studies, ATP:ADP ratio measurements, and bone marrow biopsy.
(i) A CBC will reflect a WBC count between 2,000 to 15,000/mm³ and a possible neutropenia.
(ii) Platelet counts are usually normal
(iii) Large eosinophilic, peroxidase-positive inclusion bodies in the myeloblasts and promyelocytes in the marrow
(iv) Bleeding time will be prolonged.
(v) Coagulation screening will be normal.
(vi) Blood smear examination will reveal leukocytes with darkly stained giant granulation.
(vii) Platelet aggregation profile will reveal decreased platelet aggregation in response to collagen and ADP.
(d) Prognosis and treatment of CHS in the chronic stable phase includes a multidisciplinary approach.
(i) High doses of vitamin C have been suggested to improve phagocytic function.
(ii) Treatment in the accelerated phase may include a combination of corticosteroids, vincristine, and cyclophosphamide to partially arrest the lymphohistocytic infiltration of the secondary lymphoid tissues but is not usually successful in arresting the progression of the disease.
(iii) To date, bone marrow transplantation has been successful in some, of the reversal of the accelerated phase of CHS.

(3) Wiskott-Aldrich syndrome (WAS)
(a) This disorder is a rare X-linked immunodeficiency disorder highlighted by a thrombocytopenia, eczema, recurrent infections, and a predisposition for secondary leukemia or lymphoma.
(b) The syndrome is caused by the deletion of a specific gene on the X-chromosome called the WAS protein gene (WASp). Gene expression is limited to cells of hematopoietic lineage.
(c) Although the function of WASp is not fully known as yet, there is evidence that it seems to function as a bridge between signaling and movement of the actin filaments in the cytoskeleton of hematopoietic cells such as platelets and lymphocytes.

(d) The deletion results in immunodeficiency that is caused by low production of B and T lymphocytes. Immunodeficiency is found in the majority and commonly includes IgM deficiency.

(i) Because both types of lymphs are affected, these young males are susceptible to repeated infections from bacteria, fungi, and viruses.

(ii) Ear infections, meningitis, and pneumonia are common.

(e) Diagnosis is usually made on the basis of the patient’s age and sex and the demonstration of a thrombocytopenia, a weak antibody response to certain specific polysaccharide antigens, and weak level of platelet aggregation response.

(f) The manifestations of the syndrome are localized hemorrhage, eczema, proneness to infection and bloody diarrhea.

(g) Laboratory diagnosis includes serum immunoglobulin levels, functional testing of humoral and cellular immunity, and CBC with cytoflow studies.

(i) In classic WAS, IgM levels are low and IgG levels are normal.

(ii) A CBC with manual differential is helpful, paying special attention to lymphocyte numbers and platelet size.

(iii) Patients with WAS usually have a mean platelet volume (MPV) of 3.8 to 5.0 fl compared to a normal range of 7.1 to 10.5 fl.

(iv) Counting T- and B-lymphocyte subsets by flow cytometry are useful. T-cell deficiency may occur, but many times the B-cell number is normal.

(v) Patients will demonstrate a weak response with CD43 and a low CD8 count.

(h) Prognosis and treatment with standard supportive therapy is poor. Death usually occurs before the age of 10 due to infection, malignancy, and bleeding.

(i) Standard treatments include antibiotics for infections and platelet transfusions to limit bleeding.

(ii) Immune globulin is sometimes given to strengthen the patient’s immune system.

(iii) If the thrombocytopenia becomes severe, splenectomy may be beneficial to reduce the risk of bleeding five to sixfold.

(iv) To date, the most successful form of treatment is a bone marrow transplant from a compatible sibling. Marrow transplantation may produce a cure in an appropriate HLA compatible donor is found.

(b) α-Granule deficiencies are rare platelet functional abnormalities in which both aggregation and release properties are diminished.

(1) Storage pool disease, also known as Grey platelet syndrome (GPS) is a congenital platelet disease which associates thrombocytopenia and aggregation abnormalities.

(a) Inheritance is autosomal dominate.

(b) The disorder is typically mild and patients commonly present with mild epistaxis, easy bruising, and long-lasting hemorrhages after accidental cuts.

(c) Is characterized by a marked decrease or absence of platelet α-granules and specific α-granule proteins. Morphologically, platelets appear large and contain few granules, giving them a grey appearance with Wright-Giemsa stained blood smears.

(i) Analysis of platelet contents has showed an elevated mean ATP/ADP ratio of 3.8 as compared to a normal of 1.6.

(ii) Serotonin content was also decreased in the affected platelets as compared to normal.
Aggregation studies demonstrated that patient aggregation response to ADP, collagen, or epinephrine is markedly impaired, but thrombin and arachidonic acid aggregation is normal.

(d) Laboratory diagnosis typically reveals a decreased platelet count with low MPV and abnormal aggregation results as described previously.
(ii) Other diagnostic criteria include an increased bleeding time.
(iii) The diagnosis can be confirmed with transmission electron microscope studies to reveal lack or decrease of α-granules.

(e) Prognosis of this disorder is mild and not life-threatening; treatment is commonly supportive with platelet transfusion given in rare cases of severe chronic thrombocytopenia.

3. Deficiencies of thromboxane generation can occur because of a genetic deficiency of the cyclo-oxygenase enzyme. Platelet aggregation tests are unresponsive to arachidonic acid as a stimulator.

(a) Platelet secretion of granular content depends on the activation of a series of phospholipases that catalyze the release of arachidonic acid.
(1) This metabolite is converted to intermediate prostaglandins by the enzyme cyclooxygenase, and then on to thromboxane A₂ (TXA₂) by another enzyme, thromboxane synthetase.
(2) TXA₂ functions to stimulate a cascade of events resulting in secretion and platelet aggregation by mobilization of iron calcium from internal stores into the cytoplasm.

(b) Deficiencies of thromboxane generation that occur due to a genetic deficiency of the cyclooxygenase enzyme are very rare. Only two families have been reported with a genetic TXA₂ deficiency.

c. Several acquired or genetic disorders of platelet secretion are traced to structural and functional modifications of arachidonic acid or pathway enzymes. Dysfunction or deficiency of thromboxane receptors is also known.
(1) Ingestion of aspirin and ibuprofen causes inhibition of arachidonic acid pathway enzymes; therefore, these types of disorders are referred to as having “aspirin-like effects.”

(d) In the absence of cyclooxygenase, secretion and secondary aggregation does not occur.
(1) Such deficiencies produce a platelet aggregation pattern that is similar to storage pool disease.
(2) In contrast to storage pool disorders, ultrastructural and granular contents are normal.

e. Laboratory diagnosis will find that the platelet count in such cases is normal, but the bleeding time is increased.
(1) Primary aggregation is normal.
(2) Platelet aggregation tests will be unresponsive to arachidonic acid, ADP, and collagen in the secondary aggregation stage.

(f) The prognosis of such disorders is not life-threatening and easy to manage. Patients with these deficiencies present with a mild bleeding disorder and treatment usually consists of avoiding antiplatelet drugs and in females controlling menorrhagia with hormonal therapy.

4. Acquired disorders of platelet function exist that are secondary to the following conditions:

(a) Thrombocytopenia and platelet function defects, caused by a depletion of α-granules, are seen in some patients after cardiopulmonary bypass.
(1) Functional defects result from platelet activation and fragmentation in the bypass mechanical process.
(a) Causes the bleeding time to increase more than 30 minutes.
(b) Causes of defects and granule depletion include:
   (i) Aggregation of platelets by fibrinogen absorbed onto the surfaces of the bypass circuit material
   (ii) Hypothermia
   (iii) Complement activation
   (iv) Mechanical trauma and shear stresses
   (v) Bypass pump-priming solutions
(c) Platelet fragments are commonly found in the blood of bypass patients.

Typically, after a normal bypass without complications, normal platelet function returns in about 1 to 3 hours after surgery, but platelet count of course takes several days for the marrow to correct for loss.

Thrombocytopenia can also be amplified by hemodilution as the blood passes through the bypass mechanism and removal of damaged platelets by the patients liver and spleen.

b. Disseminated intravascular coagulation (DIC) refers to a deposition of large amounts of fibrin throughout the microcirculation, which results in a pathological activation of platelet aggregation and coagulation pathways.

(1) DIC is classified as a consumption coagulopathy because it results in depletion of platelets, as well as plasma coagulation factors.
(2) Plasminogen is activated on a wider scale to plasmin, resulting in an increase in fibrin degradation products (FDP) in the plasma.
(3) Intrinsic system activation by large amounts of tissue factor entering the circulation and can result from hypofibrinogenemic states of pregnancy, metastatic carcinoma, or promyelocytic leukemia.
(4) Extrinsic system activation results from events that damage or alter the vascular endothelium, thereby exposing collagen (e.g., infectious diseases, antigen-antibody complexes, liver disease, snake venom poisoning, massive trauma, or surgery).
(5) Common diagnostic test results include:
   (a) Thrombocytopenia
   (b) Prolonged coagulation screening tests
   (c) Elevated FDP or D-Dimer
   (d) Decreased plasma fibrinogen

c. Lupus-like anticoagulant is developed in 10% to 20% of people with SLE, a significant number of patients taking the drug, phenothiazine, and occasionally in cases of lymphoproliferative disorders.
   (1) Autoantibodies found in the patient’s plasma are directed against the phospholipid portion of phospholipoprotein components found as part of the platelet membrane surface, as well as the laboratory reagent used to perform the APTT.
   (2) Occasionally patients with lupus-like anticoagulant have a mild thrombocytopenia and a functional defect of granular release.
      (a) The presence of clinical bleeding with lupus-like anticoagulant is only found in patient’s with high titers of anticoagulant.
      (b) The antibody is first detected by a slightly prolonged APTT. Further testing for a specific factor deficiency will prove to be normal.

d. Heparin-induced thrombocytopenia occurs in a significant number of patients who receive heparin demonstrate a slight transient decrease in platelet count.
   (1) The mechanism of thrombocytopenia is postulated as a direct platelet aggregating effect as well as immune destruction by antiplatelet antibodies. Sequestration by the spleen is also a factor.
   (2) Diagnostic criteria include:
      (a) Abnormal PT and APTT time
      (b) Low platelet count
      (c) Prolonged bleeding time
      (d) Positive antitheparin antibody
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(a) Normal aggregation pattern in platelet aggregometer studies, except that adding heparin as stimulant will increase aggregation instead of blunting the aggregation reaction. Symptoms subside upon the discontinuation of heparin.

e. Uremia due to kidney failure (Hemolytic-uremic syndrome) sometimes causes platelet aggregation abnormalities because of prostacyclin production and decreased platelet TXA2 production.

(1) A qualitative abnormality of platelet function is the most common finding.

(2) The defect is extrinsic and is proportional to the level of metabolite buildup in the blood due to lack of renal function.

(a) The most damaging metabolites to platelet function are thought to be urea, guanidinosuccinic acid, and phenolic acid.

(b) Endothelial cells in uremia demonstrate an increase in prostacyclin-like activity.

(c) Blood urea nitrogen (BUN) levels and bleeding time results are directly proportional and inversely proportional to the hematocrit.

(3) Diagnostic criteria include abnormal values for bleeding time and the PT. Platelet retention is abnormal and aggregation studies show abnormal results with ADP, collagen, epinephrine and ristocetin.

f. Drug induced platelet abnormalities are the most common cause of acquired platelet dysfunction.

(1) Functional inhibition is most common with the group of drugs that inhibit platelet prostaglandin production, such as aspirin. Other classifications of drugs affect platelet function by altering membrane function.

(2) Chemical compounds may alter the bleeding time and platelet aggregation studies.

(a) The most common inhibitors are aspirin, alcohol, and certain antibiotic agents such as penicillins and cephalosporins.

(b) Aspirin can result in a platelet dysfunction by irreversible inactivation of the cyclooxygenase enzyme and the resultant decrease in TXA2 formation.

(c) Other nonsteroidal anti-inflammatory drugs, such as ibuprofen-related drugs, have the same effect.

(d) Chronic ingestion of large quantities of alcohol can lead to platelet dysfunction. Studies of this dysfunction suggest a mechanism of an inhibition of prostaglandin synthesis, the alteration of the storage pool of ADP/ATP, or an alteration of platelet membrane stabilization.

(e) Antibiotics that are designed to affect bacterial cell wall synthesis can alter platelet function by decreasing the aggregation response with ADP in the primary or secondary wave of aggregation.

(3) Diagnostic criteria commonly include an increase in the bleeding time. Platelet aggregation studies usually result in a pattern of abnormal aggregation in response to ADP, collagen, and epinephrine.

(4) In the majority of cases, full platelet function returns after the offending drugs are discontinued.

III. BLOOD COAGULATION AND FIBRINOLYSIS (Figure 2–4)

A. Initiating reactions (contact activation)

1. The intrinsic system refers to the path of the coagulation cascade in which prekallikrein, heavy-molecular-weight kininogen (HMWK), and factors XII, XI, X, IX, VIII, V, II, and I are involved in the formation of a fibrin clot. In the laboratory, the APTT is used to test the coagulation cascade.

a. Initiation of the intrinsic system coagulation cascade is by the activation of factor XII (Hageman factor).

(1) Vascular damage exposes negatively charged subendothelial tissue.
The coagulation cascade. Each enzymatic factor represented by roman numerals is converted in turn to an activated form designated by the letter “a.” The intrinsic pathway consists of high-molecular-weight kininogen (HMWK); prekallikrein; and factors I, II, V, VIII, IX, X, XI, and XII. The intrinsic pathway is screened by the APTT. The extrinsic pathway consists of factors I, II, VII, and X. It is screened by the PT test. Ca^{2+}, calcium ion; PF3, platelet factor 3; (−), negative surface charge of exposed subendothelium.

2. The inactive zymogen form of factor XII is attracted to the negatively charged endothelial surface of the damaged blood vessel. The negative polarity activates factor XII by causing the molecule to expose its active serine center. The activated form of factor XII is then denoted as factor XIIa.

b. There are three products of factor XIIa reactions.

1. Prekallikrein is enzymatically cleaved by factor XIIa to produce kallikrein, which in turn produces bradykinin.
   a. Bradykinin functions to increase local vasodilatation and membrane permeability to increase local blood flow.
   b. The reaction requires HMWK as a cofactor (Figure 2–4).
Plasminogen is enzymatically cleaved by factor XIIa to functional plasmin, which initiates clot dissolution reactions.

(3) Factor XIIa enzymatically activates factor XI (plasma thromboplastin antecedent) to yield factor Xla. The activation of factor Xla will continue the coagulation cascade.

2. The extrinsic system
   a. Initiation. Contact activation of the extrinsic system begins with the activation of factor VII.
      (1) Factor III, known as tissue factor, is the primary activator of VII to VIIa, which is a potent serine protease.
      (2) Tissue factor consists of lipoproteins, which are produced in many tissues.
      (3) Minor activation of factor VII can occur by proteolytic attack from factors XIIa, Xa, IXa, or thrombin.
   b. In laboratory testing of the extrinsic system, lipoprotein-rich extracts are added to citrated plasma as the PT reagent to support the activation of factor VII by tissue factor.

B. Intermediate reactions
   1. Factor VIIla in the extrinsic pathway enzymatically alters factor X to yield Xa in the presence of factor III. Factor VIIla has limited ability to activate the conversion of IX to its activated form (IXa).
   2. Factor IX in the intrinsic pathway is most strongly activated by the direct enzymatic action of Xla.
      a. Initiation. This reaction does not require tissue factor lipoprotein as extrinsic pathway activation. It does require negatively charged membrane phospholipids and ionized calcium. Platelets are the main source of in vivo phospholipid surfaces.
      b. In laboratory testing of the intrinsic system, phospholipid extracts are added to citrated plasma as part of the APTT reagent to provide the activation for platelet-supported reactions.
   3. Factor X (Stuart-Prower factor) is activated by two different pathways.
      a. In the extrinsic pathway, factor X is enzymatically activated by VIIla, with factor III and calcium as cofactors.
      b. In the intrinsic pathway, factor X is activated by factor IXa. Factor IXa forms a complex with a platelet phospholipid membrane surface and factor VIII (antihemolytic factor) in the presence of calcium.
   4. The factor VIII complex is a high-molecular-weight (MW) complex formed of two subunits.
      a. VIII:C (antihemolytic factor) is synthesized in the liver and is genetically controlled on the X-chromosome (sex-linked transmission).
         (1) Function. Factor VIII:C serves as a cofactor in the activation of factor X by factor IXa. The presence of VIII:C accelerates the reaction rate by 500–1000 times.
         (2) Activator. Factor VIII:C is activated by thrombin.
         (3) Inactivator. Coagulation inhibitor protein C (PC) breaks down factor VIII:C enzymatically.
         (4) Pathology. An inherited deficiency of factor VIII:C is known as hemophilia A.
      b. Factor VIII:R (vWF) is synthesized by endothelial cells, megakaryocyte, and platelet and demonstrates autosomal genetic expression.
         (1) Function. vWF supports the adhesion of platelets to the exposed subendothelial surface of the blood vessel.
         (2) Activator. vWF activation occurs through the release of platelet aggregators from damaged subendothelial tissue and from the release of platelet α-granule contents.
         (3) Pathology. An inherited deficiency of factor VIII:R is known clinically as von Willebrand’s disease (vWD).
CHAPTER 2 Hemostasis and Coagulation

5. Factor Xa activation begins the common pathway, because the following enzymatic reactions are shared by both the intrinsic and the extrinsic pathways.
   a. Factor Xa enzymatically cleaves the zymogen prothrombin (factor II) to its activated form, thrombin.
   b. The activated form of factor V (proaccelerin) acts as a cofactor for factor Xa activation of prothrombin. Factor V is converted to its active form by thrombin.
   c. The combination of phospholipid membrane surface, factor Xa, factor Va, and calcium forms the receptor complex known as thrombomodulin, which supports the enzymatic conversion of prothrombin to the active enzyme thrombin (Figure 2–4).

6. Thrombin (IIa) is a powerful enzyme with many functions, including:
   a. Enzymatic conversion of fibrinogen to fibrin monomer
   b. Activation of factor XIII (fibrin stabilizing factor)
   c. Activation of platelet aggregation
   d. Activation of factor V and factor VIII:C
   e. Activation of PC
   f. Weak activation of factor VII to factor VIIa

C. Fibrin clot formation is the last step in the coagulation cascade.
   1. Thrombin enzymatically converts fibrinogen (factor I) to fibrin.
   2. Fibrinogen has the highest plasma concentration of any clotting factor, with a normal range of 150 to 400 mg/dL. The molecule is produced in the liver and has a unique molecular structure.
      a. The fibrinogen monomer consists of two identical subunits bound together to produce a symmetric structure.
      b. Three nodular domains in the fibrinogen molecule have been identified as two identical D regions at either carboxy-terminal end and a central E domain at the N-terminal end.
   3. Thrombin enzymatically activates the fibrinogen monomer by splitting off the fibrinopeptides A and B from the N-terminals in the E domain.
   4. The thrombin-exposed N-terminal peptides in the E domain react noncovalently by electrostatic forces with polar D domain regions of adjacent fibrin molecules to form a polymer structure.
   5. Formation of a fibrin polymer is the endpoint detected in the majority of in vitro clotting time tests.
   6. Clot stabilization is achieved by factor XIIIa (fibrin stabilizing factor) from the formation of covalent bonds between chains of adjacent fibrin molecules.
      a. The inactive circulating zymogen form of factor XIII is activated by the proteolytic action of thrombin with calcium and fibrinogen serving as cofactors.
      b. Factor XIIIa also covalently cross-links α2-antiplasmin to the fibrin clot, rendering the clot less susceptible to lysis by plasmin.

D. Vitamin K-dependent factors are coagulation factors (i.e., factors II, VII, IX, X) and inhibitors [i.e., PC, protein S (PS)] that depend on vitamin K metabolism to be completely functional.
   1. Without vitamin K, the coagulation factors and inhibitors are nonfunctioning, even when present in normal concentrations.
   2. Coumarin anticoagulants inhibit vitamin K reduction from the epoxide form. The end result is that factors II, VII, IX, and X are rendered inactive.
      a. Unlike heparin, coumarin is inactive as an in vitro anticoagulant and functions only as a therapeutic in vivo anticoagulant.
      b. The PT test is the best screening method for coumarin therapy because factor VII has the shortest half-life and is the most sensitive to levels of coumarin therapy.

E. Natural inhibitors of coagulation function to counterbalance the effects of coagulation factors, provide limitations for the forming fibrin clot, and prevent systemic thrombus formation.
1. **Antithrombin III** is the principal inhibitor of thrombin and factor Xa with limited inhibitory activity against factors IXa, XIa, and XIIa.
   a. **Antithrombin III** functions by binding with thrombin to form a 1:1 inactive complex.
   b. **Heparin** serves as a cofactor in the inactivation, thereby increasing the reaction rate by more than 2,000 times.

2. **α₂-macroglobulin** is a minor inhibitor of thrombin.
3. **Complement C₁ inhibitor** is a minor inhibitor of factors XIa and XIIa.
4. **α₁-antitrypsin** has limited inhibition of thrombin, kallikrein, and factor Xa.
5. **PC** is a vitamin K-dependent inhibitor that circulates as an inactive zymogen.
   a. **Activator.** PC is activated by thrombin as part of the thrombomodulin platelet receptor complex.
   b. **Function.** PC inactivates factors VIII: C and Va in the presence of cofactor PS. PS also depends on vitamin K, functions to enhance binding of PC to phospholipid surfaces, and increases the rate of Va and VIIIa inactivation by PC.

F. **Laboratory testing of coagulation depends on the quality and freshness of the plasma specimen obtained.** Whole blood anticoagulated with sodium citrate is the specimen of choice. A 9:1 blood:citrate ratio is required for accurate coagulation testing, because a ratio of <9:1 may falsely increase results. Conditions that can interfere with obtaining the required 9:1 ratio are an abnormally high hematocrit, traumatic blood drawing, or a hemolyzed specimen. **EDTA contamination** can falsely increase PT and APTT results. Specimens must be assayed as soon as possible, and the plasma must be kept cold to avoid factor deterioration.

1. **PT tests for extrinsic pathway deficiencies in factors VII, X, V, II, and I (fibrinogen).**
   a. **Reagent.** A lipoprotein tissue extract from brain or lung tissue serves as the reagent source of tissue factor. An excess of calcium is also added to the PT reagent.
   b. **Principle.** Citrated plasma is added to the lipoprotein reagent with calcium, and the time required for fibrin clot formation is measured.
   c. **Reference range.** Although the PT assay has an approximate normal range of 11 to 13 seconds, it is important for each laboratory to establish its own range.
   d. **Variation.** The addition of Russell’s viper venom (Stypven) instead of lipoprotein reagent activates factor X directly, bypassing factor VII as a necessary component variable. This variation of the common PT is known as the Stypven time.

2. **The international normalized ratio (INR)** by definition is the PT ratio that reflects the results that would have been obtained if the World Health Organization (WHO) international reference preparation (IRP) thromboplastin had been used to perform the test.
   a. The specific purpose of the INR is for reporting results on stable, orally anticoagulated patients taking Coumarin.
   b. **Calculation of the INR.**
      (1) In order to calculate the INR, it is necessary to have a PT ratio and the international sensitivity index (ISI) value of the thromboplastin reagent used to measure the PT.
      (2) The ISI value is assigned by the manufacture of the thromboplastin reagent, and attests to the purity of the reagent. The lower the manufacture ISI value, the more desirable the reagent for use. An ISI value of 1.0 would claim equal purity with the WHO thromboplastin IRP.
      (3) The PT ratio is calculated as follows:

      \[
      \text{INR} = \frac{\text{patient PT}}{\text{mean of normal PT}}^\text{ISI} 
      \]

(4) The INR is then derived by the following formula:

\[
\text{INR} = \{\frac{\text{patient PT}}{\text{mean of normal PT}}\}^{\text{ISI}}
\]
log (INR) = ISI × log (patient PT/normal PT)

c. Therapeutic ranges of INR:
(1) Patients not on oral anticoagulants with normal coagulation should have low PT values in the range of the INR.
(2) The majority of patient’s INRs of 2.0 to 3.0 corresponds to PT ratio for thromboplastin of 1.5 to 2.0.
(3) When the INR is correctly used for monitoring a patient’s oral anticoagulant therapy, the physician gives a standard dose of coumarin to achieve a target INR between 2.0 and 3.0.
(4) If the physician chooses to use a high dose of coumarin, the target value INR is between 2.5 and 3.5. Indications of high-dose anticoagulant therapy are mechanical heart valves.
(5) An INR value >3.5 constitutes a panic value and should be reported to the patient’s nurse immediately.

3. APTT tests for intrinsic pathway deficiencies in factors prekallikrein, HMWK, and factors XII, XI, IX, X, VIII, V, II, and I.

a. Reagents. A phospholipid-rich preparation is used as a platelet-membrane substitute. An activator, such as kaolin, ellagic acid, or celite, is also added to the APTT reagent to provide the negative surface charge required to activate factor XII and prekallikrein. Calcium chloride is used as an additional reagent to initiate clotting.

b. Principle. A citrated plasma sample is preincubated with the phospholipid reagent to initiate contact activation factors in the intrinsic pathway. Following incubation, calcium chloride reagent is added as a separate reagent to initiate the clotting cascade. The time required for fibrin clot formation to occur is measured.

c. Reference range. The APTT assay has an approximate normal range of 25 to 40 seconds, but it is important for each laboratory to establish its own range.

4. Thrombin clotting time (TCT) tests for a deficiency or inhibition of fibrinogen.

a. Principle. Commercially prepared thrombin reagent is added to citrated plasma, and the time required for clot formation is measured.

b. Reference range. The range is generally 10 to 20 seconds, but each laboratory should establish its own range.

c. A prolonged TCT can occur in patients receiving therapeutic heparin, patients with increased fibrin degradation products, and patients with any disorder of hypofibrinogenemia.

5. Quantitative fibrinogen assay is an expansion of the TT methodology.

a. Principle. A measured amount of commercially prepared thrombin reagent is added to citrated plasma. The clotting time is measured and compared with the clotting times of plasma fibrinogen standards containing known amounts of fibrinogen.

b. A standard curve is constructed, and the clotting time in seconds is plotted against milligrams per deciliter of fibrinogen. Patient unknown data can be quantitated for fibrinogen from a standard curve.

c. Reference range. The normal range for a quantitative fibrinogen is 200 to 400 mg/dL.

6. Substitution tests (Table 2–3) can be adapted if primary tests like the PT or APTT are abnormally prolonged and indicate a factor deficiency. The patient’s deficient plasma is diluted 1:1 with a plasma or serum substitute, and the APTT or PT is repeated. A correction of the original prolonged APTT or PT indicates that the deficient factor had been added to the patient’s plasma by the substitution solution. The prepared substitution solutions are as follows:

a. Aged plasma lacks labile factors V and VIII, but retains normal activity of all other coagulation factors. Normal plasma retains normal activity of all coagulation factors.

b. Fresh absorbed plasma lacks vitamin K factors (i.e., factors II, VII, IX, X), but retains normal activity of all other coagulation factors.
A Concise Review of Clinical Laboratory Science

Table 2–3 Substitution Testing with Mixing Studies∗

<table>
<thead>
<tr>
<th>Intrinsic Pathway PT</th>
<th>Extrinsic Pathway APTT</th>
<th>Factor-deficient Plasma or Serum</th>
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</thead>
<tbody>
<tr>
<td>Normal Plasma</td>
<td>Adsorbed Plasma</td>
<td>Aged Serum</td>
</tr>
<tr>
<td>I</td>
<td>I</td>
<td>+</td>
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<tr>
<td>XII</td>
<td>XII</td>
<td>+</td>
</tr>
</tbody>
</table>

APTT = activated partial thromboplastin time; (−) = factor missing; + = factor present; PT = prothrombin time.

∗By using the PT and APTT screening tests and mixing patient plasma samples with known factor deficient plasma, the majority of coagulation factor deficiencies can be determined. Each factor deficiency will result in a specific testing pattern.

c. Aged serum lacks factors I, II, V, and VIII, but retains normal activity of all other coagulation factors.

7. Final confirmation and quantitation of a factor deficiency is done with specific factor assays. These methods use a test plasma with a known deficiency, which is titrated and tested against the patient’s plasma unknown factor deficiencies. Factors can also be immunologically assayed with enzyme linked immunosorbent assay (ELISA) methodology.

8. Chromogenic assays constitute methods that use a synthetic substrate targeted to be enzymatically altered by a specific serine protease or serine protease inhibitor in a patient’s plasma specimen.
   a. Principle. The specific substrate is cleaved by the targeted serine protease factor in the plasma sample to yield a chromogenic (colored) or a fluorogenic compound.
   b. Measurement. An endpoint reaction yields a color whose intensity is directly proportional to the activity of the serine protease. The color intensity can be measured on a spectrophotometer and quantitated with a standard curve.

9. The activated clotting time (ACT) is a modification of the whole-blood clotting time test used to monitor heparin therapy.
   a. The principle is based on the mixing of a specified amount of particulate clot activator with whole blood.
      (1) A timer is started while the blood is being continuously mixed until a clot is formed.
      (2) The ACT may be performed manually or with an automated ACT timer, such as a Hemoschron instrument.
   b. The average normal ACT is 98 to 100 seconds.
      (1) Heparin is given therapeutically to yield an ACT range of 180 to 240 seconds in deep vein thrombosis.
      (2) For patients undergoing cardiopulmonary bypass, a therapeutic range of heparin should result in ACT times around 400 seconds.
   c. The ACT is better suited than the APTT in measuring therapeutically high doses of heparin.

10. Reptilase time (RT) is a modification of the TCT.
    a. Reptilase is the venom of Bothrops atrox, which acts like a thrombin-like enzyme to catalyze the conversion of fibrinogen to fibrin.
    b. The venom cleaves only the fibrinopeptide A from the fibrinogen molecule.
    c. A prolonged reptilase time indicates a functional fibrinogen problem.
       (1) The advantage of the RT over the TCT is that Reptilase is insensitive to the effects of heparin and sensitive to dysfibrinogenemia and hypofibrinogenemia.
       (2) The RT is also prolonged in the presence of FSP.
11. Factor XIII Screening is a simple test using urea to dissolve clots.
   a. Factor XIII is responsible for converting the fibrin clot to a stable form.
   b. It is activated by thrombin during the fibrinogen-to-fibrin conversion.
   c. When factor XIII is present, a fibrin clot is insoluble in 5 M urea when left standing for 24 hours. If factor XIII is deficient, 5 M urea will dissolve a fibrin clot within 2 hours.

12. Lupus-like anticoagulant/antiphospholipid antibodies is developed in 10% to 20% of people with SLE, a significant number of patients taking the drug, phenothiazine, and occasionally in cases of lymphoproliferative disorders.
   a. Inhibitors most commonly are IgG and occasionally IgM. Autoantibodies found in the patient’s plasma are directed against the phospholipid portion of phospholiprotein components found in APTT reagent.
   b. Occasionally patients with lupus-like anticoagulant have a mild thrombocytopenia.
   c. The presence of clinical bleeding with lupus-like anticoagulant is only found in patient’s with high titers of anticoagulant.
   d. The antibody is first detected by a slightly prolonged APTT with a normal PT and TT.
      (1) The APTT will not be corrected with a 1:1 dilution of patient and normal plasma like a typical factor deficiency.
      (2) Increasing prolonged times are directly proportional to extended incubation times with the APTT phospholipid reagent.
   e. Further testing for a specific factor deficiency will prove to be normal.

13. Factor VIII assay and other specific factor assays are based on the ability of commercially prepared plasmas to correct a factor-deficient patient.
   a. The APTT assay is used to estimate the concentration of functional factor VIII by serial diluting the patient’s plasma with a VIII-depleted plasma control.
      (1) Factor VIII-depleted plasma contains full activity of all factors except VIII, which has been immunodepleted.
      (2) The factor-depleted plasma will have a prolonged APTT unless corrected by diluting with a normal plasma control.
      (3) A prolonged APTT result on a mixture of the patient plasma and the factor-deficient plasma implies that the patient is deficient in the same factor that is missing in the factor-depleted control.
   b. The APTT clotting time interval of a 1:10 dilution of the patient plasma and the factor-depleted plasma is compared to a previously prepared reference curve to obtain the % activity of the factor plasma.
      (1) The reference graph is prepared with a series of five or more dilutions of factor-depleted control plasma with a normal coagulation control.
      (2) The reference curve is plotted with clotting time on the y-axis against % activity of factor VIII, or another factor, on the x-axis.

14. Point of care testing (PCT) in coagulation has been developed for bedside testing of the PT, APTT, TCT, and the ACT to provide rapid turn-around-times for the physician.
   a. An automated device such as the Hemochron Portable Blood Coagulation Timing System may be used.
   b. The methodology uses a technique similar to the automated ACT.
      (1) Commercially prepared tubes containing thromboplastin and a magnetized stir bar are used for the PT.
      (2) Tubes containing diatomaceous earth (for an activator), partial thromboplastin, buffer, calcium chloride, and a magnetized stir bar are used for the APTT.
      (3) Tubes containing lyophilized thrombin, buffer, calcium chloride, and a magnetized stir bar are used for the TCT.
   c. Two milliliters of whole blood is collected and transferred to the reaction tube.
      (1) The timer is started after the tube is placed in the reaction well.
      (2) Whole blood PT and APTT results are interpreted by blood-to-plasma conversion tables provided by the manufacturer.
G. Normal fibrinolysis refers to the enzymatic pathway of clot dissolution in which plasmin is the key enzyme responsible for breaking down bonds in the fibrin polymer and releasing fibrin degradation products (FDP).

1. Plasmin activation results from proteolytic cleavage of a circulating inactive zymogen known as plasminogen by either of two pathways:
   a. Extrinsic pathway activation in vivo involves a tissue proteolytic enzyme and fibrin as a cofactor.
      (1) Urokinase can also convert plasminogen to plasmin and is used as a thrombolytic agent.
      (2) Streptokinase is a streptococcal-derived protein used as a therapeutic thrombolytic drug.
   b. Intrinsic pathway activation of plasmin involves factors XIIa, kallikrein, or HMWK.

2. Plasmin degradation of fibrin begins by the breaking down of the fibrin polymer into a monomer form known as fragment X. This fragment is identical to a fibrin monomer, consists of one E domain and two D domains, and retains clotting properties.
   a. Fragment X is further cleaved to produce FDP, which consists of:
      (1) Fragment Y, which consists of one E domain and one D domain
      (2) Fragment E, which consists of the E domain only
      (3) Fragment D, which consists of the D domain only
      (4) D dimer, which consists of two D fragments of linked monomers
   b. The FDPs produced during fibrinolysis have anticoagulating effects of their own because they inhibit the process of fibrin polymerization, demonstrate competitive inhibition of thrombin, and prolong the TT test.

3. Other functions of plasmin in addition to fibrin degradation include:
   a. Fibrinogen degradation
   b. The inactivation of factor VIIIa
   c. The inactivation of factor V
   d. Direct antiaggregation effect on platelets

4. α2-Antiplasmin inhibits the actions of plasmin by forming a 1:1 complex with plasmin, which prevents fibrin binding.
   a. α2-Antiplasmin is bound to the fibrin by factor XIIIa.
   b. α2-Macroglobulin also has weak antiplasmin activity.

H. Laboratory tests for fibrinolysis

1. Latex agglutination can measure freely circulating FDP in the patient’s serum. Latex coated with antifibrinogen detects the presence of increased levels of FDP. Only serum can be used to avoid interference with endogenous fibrinogen.

2. Plasminogen or α2-antiplasmin can be measured serologically or with chromogens.

3. D dimer test is based on a highly specific, monoclonal antibody directed against a unique neoantigen of covalently crosslinked D fragments resulting from fibrinolysis. The D dimer has several advantages in detecting degradation fragments when compared with the standard FDP assay.
   a. The FDP assay can be run on plasma, eliminating the risk of false positives.
   b. D dimer is superior in sensitivity and specificity as compared with the conventional FDP assay (i.e., sensitive to as little as 20 ng/mL, as compared with 10 μg/mL for conventional latex FDP).

4. Prothrombin fragment 1.2 (F-1.2) is a new assay that tests for the presence of the fragment generated when prothrombin is activated to thrombin (factor IIa).
   a. Prothrombin fragment serves as a sensitive biologic marker of thrombin generation and Xa activity because generation of F-1.2 precedes thrombus formation.
   b. Levels are elevated in persons predisposed to thrombotic risk (e.g., cancer, heart disease, orthopedic surgery).
   c. Levels are depressed in persons undergoing anticoagulant therapy.

5. Euglobulin Lysis Time is used as a screening test for fibrinolytic activity.
   a. Euglobulin fraction of plasma consists of plasminogen, fibrinogen, and activators of plasminogen.
b. The euglobulin fraction is precipitated with 1% acetic acid and resuspended in a borate solution.
   (1) Euglobulins are clotted by the addition of thrombin (5 units/mL).
   (2) The resulting clot is incubated and the time of lysis is obtained.

c. Clot lysis in <2 hours is indicative of abnormal fibrinolytic activity.
   (1) A decreased time below normal is also caused by a decrease in fibrinogen.
   (2) A prolonged time >4 hours is caused by a decrease in plasminogen or activator.

6. Protein-C (PC), and protein-S (PS), and antithrombin-III (ATIII) assays can all be measured for degree of activity with chromogenic assays or with immunoassays to measure the concentration of the molecule (i.e., antigen).
   a. PC can be accurately measured by chromogenics or enzyme immunoassay, but the latter has a better shelf life.
      (1) In the chromogenic assay, the patient plasma sample is mixed with venom to activate PC.
      (a) Added chromogenic substrate is hydrolyzed by the APC.
      (b) The amount of the hydrolyzed colored product is proportional to the activity of PC in the patient’s sample.
      (2) The immunoassay method is based on using rabbit-derived antihuman PC-coated microtiter plate wells that capture the PC in the added plasma sample.
         (a) Bound PC is conjugated with peroxidase, yielding a color product the intensity of the PC in the sample.
   b. PS can be measured with similar methodologies.
   c. ATIII can be measured with chromogenics or immunoassay using similar methodology for each.
      (1) The chromogenic uses heparin as an ATIII activator.
      (2) Activated ATIII then binds with a serine protease, such as factor X, and conjugated with peroxidase to yield the colored end product.

7. Activated protein-C resistance (APCR) is defined as the inability of APC to prolong clotting tests when added to the APTT.
   a. This phenomenon is characteristic of a genetic thrombotic disorder known as factor V Leiden.
      (1) When APC is added to normal test plasma, inactivation of factors VIII and V result in the prolongation of the APTT.
      (2) Patients with APCR demonstrate a diminished ability to inactivate V when APC is added to the test sample.
   b. The assay method is based on the prolongation of the APTT when APC is added to the test plasma.
      (1) The aPTT reagent is added to two aliquots of the patient’s plasma and preincubated.
      (2) Calcium chloride is added to one aliquot and the time to clot is recorded.
      (3) To the second aliquot, a mixture of calcium chloride and APC is added and clot formation is timed.
      (4) In a normal plasma without APCR, the APTT results of the first sample aliquot without APC should be more than twice the clotting time of the second aliquot with APC.
      (5) If the patient has a genetic resistance to PC inactivation of V, then the second APTT is shortened in time and will be less than twice the clotting time of the sample without APC.

1. Vascular factors in coagulation
   1. Blood vessels are lined with a continuous monolayer of endothelial cells anchored to inert basement membrane by a subendothelial matrix.
   2. In arteries and arterioles, the subendothelial layer is surrounded by layers of smooth muscle cells and adventitia with fibroblasts.
   3. When vascular subendothelium is exposed by vessel damage, platelet aggregation and coagulation can be directly initiated by collagen release and indirectly by tissue-released vWF.
4. Platelet-derived growth factor (PDGF) released from platelet granules stimulates the growth and migration of smooth muscle cells, endothelium, and fibroblasts to heal the wound.

5. Tears in larger vessels with surrounding smooth muscle cells have enhanced coagulatory effects.
   a. Both clotting pathways are activated by the release of tissue factor III (extrinsic pathway) and the activation of Hageman factor XIIa (intrinsic pathway) by exposed endothelium.
   b. PGI₂ is released from smooth muscle cells to limit and localize clotting.

6. Capillaries do not have a smooth muscle layer, and veins have very little, so clotting occurs primarily by platelet aggregation and activation of the extrinsic pathway. Tissue factor III is present, but activators from subendothelium and smooth muscle are minimal.

IV. COAGULATION DISORDERS (Figure 2–5)

A. Disorders of fibrinogen and related disorders
   1. Hereditary disorders of fibrinogen can be caused by quantitative or qualitative abnormalities of either fibrinogen or fibrin-stabilizing factor (factor XIII). In most of the disorders, the APTT, PT, and TT tests are prolonged.
   a. Afibrinogenemia is a quantitative deficiency of fibrinogen caused by a lack of synthesis by the liver.
Severe hemorrhages predominate in the umbilical, mucosal, gastrointestinal (GI), and intracranial regions.

The most common treatment is replacement therapy with cryoprecipitate or fresh frozen plasma (FFP) to raise the blood fibrinogen level higher than 60 mg/dL.

**b. Dysfibrinogenemia** is a qualitative abnormality in the structure and function of the fibrinogen molecule.

1. Fibrinogen levels may be normal, and the bleeding time is usually normal.
2. Posttraumatic or postoperative bleeding of mucosal tissues is common.

**c. Factor XIII deficiency** can be clinically severe with moderate-to-severe bleeding.

1. The APTT, PT, and TT tests are normal.
2. Low factor XIII concentrations are detected by incubation of a fibrin clot in a 5 M urea solution. A normal fibrin clot will not be dissolved in 5 M urea after 24 hours, but a clot deficient in factor XIII will be dissolved after 24 hours.

## 2. Acquired disorders of fibrinogen occur secondary to other pathologic events.

### a. DIC refers to a deposition of large amounts of fibrin throughout the microcirculation that results in a pathologic activation of coagulation pathways. DIC can be fatal, self-limiting, acute, or chronic.

1. **Classification.** DIC is classified as a consumption coagulopathy, because it results in a depletion of platelets as well as plasma coagulation factors.
2. **Physiologic effects.** Plasminogen is activated systemically to plasmin, resulting in an increase in FDP in the plasma. There is possible red blood cell (RBC) fragmentation caused by damage from multiple thromboemboli.

3. **Common coagulation test results include:**
   - A decreased platelet count
   - A prolonged PT, APTT, and TT
   - An elevated FDP or D dimer
   - A decreased fibrinogen

4. The causes of DIC are widespread and can occur from intrinsic or extrinsic pathway activation.
   - **Extrinsic system activation** occurs by large amounts of tissue factor entering the circulation, and can result from hypofibrinogenemic states of pregnancy, metastatic carcinoma, or promyelocytic leukemia.
   - **Intrinsic system activation** results from events that damage or alter the vascular endothelium, thereby exposing collagen (e.g., infectious diseases, antigen-antibody complexes, liver disease, snake venom poisoning, massive trauma, or surgery).

5. **Treatment** for DIC varies with the cause, but most often FFP-pooled platelet, cryoprecipitate, or low-molecular-weight heparin is used to treat the symptoms and break the cycle.

### b. Primary fibrinolysis is symptomatically similar to DIC, but results from increased levels of plasmin.

1. **Common causes include** cirrhosis, shock, metastatic carcinoma of the prostate, injury to the genitourinary tract, and leaking of urokinase from the urine into tissues.

2. **Common coagulation test results** include decreased fibrinogen, prolonged PT or APTT, and increased FDP or D dimer. In contrast to DIC, primary fibrinolysis usually demonstrates a normal platelet count, and the RBC morphology does not show fragmentation.

3. A test tube sample from a patient with primary fibrinolysis will initially form a clot that dissolves in 1 to 2 hours.

### c. Liver disease can be associated with coagulation disorders because the majority of the coagulation factors are synthesized in the liver.

1. A decrease in factor VII occurs first because factor VII has the shortest half-life of the coagulation factors.
Table 2–4 Comparison of the Thrombin and Reptilase Times to Distinguish Inherited Fibrin

<table>
<thead>
<tr>
<th>Thrombin Time</th>
<th>Reptilase Time</th>
<th>Defect</th>
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<tbody>
<tr>
<td>Infinitely prolonged</td>
<td>Infinitely prolonged</td>
<td>Dysfibrinogenemia</td>
</tr>
<tr>
<td>Infinitely prolonged</td>
<td>Equally prolonged</td>
<td>At fibrinogenemia</td>
</tr>
<tr>
<td>Prolonged</td>
<td>Normal</td>
<td>Hypofibrinogenemia</td>
</tr>
<tr>
<td>Prolonged</td>
<td>Slight to moderate</td>
<td>Fibrin degradation product</td>
</tr>
</tbody>
</table>

*Reptilase activates fibrinogen in a manner similar to thrombin but is not sensitive to heparin and fibrin degradation product inhibition.

(2) In patients with severe liver disease, the PT usually is prolonged; in later stages, the APTT will also be prolonged.

(3) Severe liver disease often causes decreased fibrinogen production (hypofibrinogenemia) or an abnormal fibrinogen molecule (dysfibrinogenemia).

d. Therapeutic heparin administration, commonly used in post-surgical and cardiac patients, can prolong the APTT and TT acutely and can prolong the PT with chronic use. In a patient receiving heparin, abnormal coagulation results must be distinguished from similar coagulopathies if the patient history is unknown. This can be done with the reptilase-R time test (Table 2–4).

B. Inherited deficiencies of other factors in the intrinsic and extrinsic pathways

1. Hemophilia A is an inherited deficiency or dysfunction of factor VIII:C (anti-hemolytic factor).

a. Inheritance pattern is sex-linked (i.e., on the X chromosome), and female homozygotes with a hemophilic father and carrier mother are very rare.

b. There are three patterns of severity.

(1) Severe hemophilia A is diagnosed in patients with spontaneous hemorrhages and a factor VIII:C level <1% of the normal level.

(2) Moderate hemophilia A is diagnosed in patients who have VIII:C levels <5% of the normal level.

(3) Mild hemophilia A is seen in patients with 6% to 30% of the normal VIII:C levels. Typically, these patients only bleed excessively in association with trauma or surgical procedures.

c. Human alloantibodies to VIII:C are produced in a minority of severe hemophilia A cases.

(1) Approximately 10% of severe hemophilics are positive for cross-reacting material (CRM+). CRM is an antigen that neutralizes anti-VIII:C antibodies.

(2) Approximately 90% of hemophilics are negative for CRM.

d. Patients with moderate-to-severe hemophilia A demonstrate a clinical bleeding pattern of spontaneous bleeding into joints or muscles, excessive postoperative hemorrhage, and easy bruising.

e. Predicted coagulation profile results include:

(1) Normal bleeding time (usually)

(2) Normal PT and TT

(3) Prolonged APTT if VIII:C levels are <0.2 U/mL (i.e., <20% activity)

(4) Female heterozygote carriers will have 25% to 75% VIII:C activity that will not usually show up on laboratory testing.

f. Treatment of hemophilia A is based on replacement therapy.

(1) Cryoprecipitate is a plasma-fraction preparation prepared by thawing FFP at 4 C. It contains a concentrated portion of fibrinogen and VIII complex.

(2) Preparations rich in factor VIII:C are more costly, but are effective.

2. Hemophilia B (Christmas disease) is an inherited deficiency in factor IX resulting from a sex-linked (X-chromosome) mutation.

a. Diagnosis. Hemophilia B is less common than hemophilia A. However, clinical symptoms are virtually indistinguishable, and laboratory differential diagnosis is necessary.
b. Treatment. A correct laboratory diagnosis must be made because treatment of hemophilia B is different than treatment of hemophilia A.

(1) Cryoprecipitate is deficient in factor IX, but rich in the factor VIII complex.

(2) FFP replacement therapy is the treatment of choice for hemophilia B, because FFP contains active factor IX. However, it is deficient in factor VIII.

(3) The half-life of factor IX (24 hours) is longer than that for factor VIII (12 hours), so the administration of treatment for a factor IX deficiency is less frequent.

c. Coagulation profile results initially may not produce results that differ from a patient with hemophilia A because the bleeding time, PT, and TT will also be normal, and the APTT will be prolonged. If hemophilia is suspected, and the APTT is prolonged, substitution tests or assay for factors IX and VIII activity should be performed.

3. vWD occurs almost as frequently as hemophilia A.

a. Genetically, vWD is inherited as an autosomal dominant condition.

b. The mutation is generally a defect in the VIII:R component of factor VIII, but partial deficiencies in the VIII:C portion often occur.

c. Patients with vWD characteristically bleed from mucous membranes and subcutaneous tissues.

(1) Easy bruising and GI bleeding is common.

(2) Muscle hematomas and deep vessel hemorrhages are not as common as in hemophilia A.

d. Production of VIII:R and VIII:C are controlled by different tissues but circulate as a VIII:C/VIII:R complex.

e. The vWF molecule consists of a basic monomer form, but it also combines with additional monomers to produce a series of multimers of increasing molecular weight.

(1) Largest MW multimers possess the functional activities contributing to platelet-mediated homeostatic events.

(2) Antigenically, there appears to be a plasma vWF that is synthesized from a tissue source and a platelet-derived vWF based on multimer size.

(3) The variation of vWF size results in several clinical subclassifications of this disorder.

(a) Clinical Type I vWD is the most common “classic” form of vWD, in which a partial decrease of all sizes of vWF MW multimers occurs.

(b) Clinical Type II vWD patients have a selective absence of higher MW multimers.

(i) In patients with Type IIa vWD, ristocetin-induced activity is decreased because both platelet and plasma vWF are absent.

(ii) In patients with Type IIb vWD, the ristocetin-induced activity is normal or increased because only plasma high-MW multimers are decreased.

(c) Patients with Type III vWD suffer from the most rare and severe type, because factor VIII:C is almost nondetectable.

(d) Some patients are classified as having a platelet-type variant, which is similar to Type IIb, but is accompanied by a thrombocytopenia.

f. The common laboratory coagulation results for patients with vWD include:

(1) Prolonged bleeding time and increased petechiae, because platelet adhesion to capillaries is impaired

(2) Normal PT time

(3) Prolonged APTT test, only if VIII:C activity is < 20%

(4) Platelet aggregation reactions that are normal with ADP, epinephrine, collagen, or thrombin activation, but there is impaired ristocetin-induced aggregation

g. Treatment. Factor VIII concentrations are not useful, because they contain primarily factor VIII C and are deficient in high MW multimers of vWF. The treatment of choice, therefore, is cryoprecipitate.
The clinical response to treatment in a patient with vWD differs from treatment of a patient with hemophilia A. The increase in VIII:C levels following treatment in hemophilia A patients is directly proportional to the amount of cryoprecipitate infused. This relationship is not found with the treatment of patients with vWD.

(a) Cryoprecipitate infusion given to patients with vWD stimulates production of VIII:C, because the defect is not in VIII:C, but in VIII:R. (b) Blood levels of VIII:C activity slowly peak 4 to 24 hours after treatment to blood levels greater than the amount contained in the infused cryoprecipitate.

4. Deficiencies of factor XII, HMWK, prekallikrein, or factor XI are rare and genetically autosomal recessive.
   a. Factor XI deficiencies have the highest incidence in the Jewish population.
   b. These bleeding disorders are clinically mild, and severe bleeding is not usually observed. Bleeding is more commonly noted, however, with factor XI deficiencies.
   c. Common laboratory coagulation results reflected by these deficiencies include:
      (1) Prolonged APTT
      (2) Normal PT, TT, and bleeding time
      (3) Normalization of the APTT results following a prolonged incubation of the patient’s plasma with an activating factor, such as kaolin (observed with a prekallikrein deficiency)

5. Factor VII deficiencies are rare autosomal recessive disorders that are not usually associated with a serious clinical bleeding history. Common laboratory coagulation results from a patient with a factor VII deficiency include:
   a. Normal APTT, TT, and bleeding time
   b. Prolonged PT but a normal Stypven time

6. Factor X deficiencies are rare autosomal recessive disorders that can be caused by quantitative and qualitative abnormalities of the factor X molecule. The expected laboratory coagulation profile includes:
   a. Prolonged PT and APTT
   b. Normal TT and bleeding time

7. Factor V deficiencies are rare autosomal recessive disorders.
   a. The level of factor V activity in the patient’s plasma does not directly correlate to the patient’s clinical severity.
   b. Approximately one third of patients with factor V deficiency have an increased bleeding time because of the platelet-related function of factor V of binding factor X to the platelet surface.
   c. The common laboratory coagulation results include:
      (1) Prolonged APTT and PT times
      (2) Normal TT

8. Prothrombin (factor II) deficiencies are extremely rare. The common laboratory coagulation profile includes:
   a. Prolonged PT and APTT, which can be corrected in substitution testing only by fresh normal plasma
   b. Normal fibrinogen assay

C. Acquired coagulation disorders (Table 2–5)
   1. Acquired disorders of factor VIII are primarily caused by the presence of autoimmune inhibitors of factor VIII.
      a. Inhibitors to VIII:C occasionally develop in normally healthy women after childbirth and disappear after a few months.
      b. Acquired VIIIIR (vWD) is seen in people with autoimmune disease or lymphoproliferative disorders.
   2. Acquired inhibition of factor XIII occurs following drug therapy with a tuberculosis drug known as isoniazid.
   3. Factor X deficiencies occur rarely in persons with an autoimmune disease known as amyloidosis.
Table 2–5

<table>
<thead>
<tr>
<th>PT Normal</th>
<th>PT Abnormal</th>
</tr>
</thead>
<tbody>
<tr>
<td>APTT normal</td>
<td>Factor XIII deficiency (H)</td>
</tr>
<tr>
<td></td>
<td>Extrinsic pathway disorder</td>
</tr>
<tr>
<td></td>
<td>Factor VII deficiency (H)</td>
</tr>
<tr>
<td></td>
<td>Early liver disease (Ac)</td>
</tr>
<tr>
<td></td>
<td>Coumarin drugs (Ac)</td>
</tr>
<tr>
<td>APTT abnormal</td>
<td>Intrinsic pathway disorders (H)</td>
</tr>
<tr>
<td></td>
<td>With clinical bleeding: Factor VIII, IX, XI</td>
</tr>
<tr>
<td></td>
<td>Deficiencies of factors, I, II, V, X</td>
</tr>
<tr>
<td></td>
<td>Liver disease (Ac)</td>
</tr>
<tr>
<td></td>
<td>Disseminated intravascular coagulation (Ac)</td>
</tr>
<tr>
<td></td>
<td>Vitamin K deficiency (Ac)</td>
</tr>
<tr>
<td></td>
<td>Lupus anticoagulant (Ac)</td>
</tr>
<tr>
<td></td>
<td>Heparin (Ac)</td>
</tr>
<tr>
<td></td>
<td>Factor VIII inhibitors (Ac)</td>
</tr>
</tbody>
</table>

Ac = acquired; APTT = activated partial thromboplastin time; H = hereditary; PT = prothrombin time.

4. Lupus-like anticoagulant is developed in 10% to 20% of persons with SLE, in a significant number of patients taking phenothiazine, and occasionally in individuals with lymphoproliferative disorders.
   a. Inhibitors most commonly used are immunoglobulin G (IgG) and occasionally IgM. Autoantibodies found in the patient’s plasma are directed against the phospholipid portion of phospholipoprotein components found in APTT reagent.
   b. Patients with lupus-like anticoagulant occasionally have a mild thrombocytopenia.
   c. The presence of clinical bleeding with lupus-like anticoagulant is found only in patients with high titers of anticoagulant.
   d. The antibody is first detected by a slightly prolonged APTT, with a normal PT and TT.
      (1) The APTT will not be corrected with a 1:1 dilution of patient plasma and normal plasma as in a typical factor deficiency.
      (2) Increasing prolonged times are directly proportional to extended incubation times with the APTT phospholipid reagent.
   e. Further testing for a specific factor deficiency proves to be normal.

5. A vitamin K deficiency results in impaired synthesis of factors II, VII, IX, X, PC, and PS.
   a. Causes of vitamin K deficiency are varied and include:
      (1) Inadequate diet
      (2) Biliary obstruction
      (3) Intestinal malabsorption diseases
      (4) Gut sterilization by chronic antibiotic therapy
      (5) Hemorrhagic disease of the newborn, which is caused by a slowly developing liver function and corrected by administration of vitamin K1
      (6) Coumarin therapy, which inhibits vitamin K metabolism and, therefore, the vitamin K-dependent coagulation factors
   b. Expected laboratory coagulation profile includes:
      (1) Acute prolonged PT (and possibly APTT) with chronic deficiency
      (2) Normal TT and fibrinogen

6. Heparin binds antithrombin III, greatly enhancing its ability to bind and inactivate thrombin.
   a. Exogenous heparin therapy is a fast and potent form of anticoagulation.
   b. Heparin is commonly used to treat thrombosis. Many patients receive heparin for a short period to prevent emboli after major surgery.
   c. The APTT is the most commonly used test to monitor heparin therapy, but the TT is also prolonged.
   d. Occasionally, the PT is prolonged if the patient has received heparin for a long period.
Figure 2–6 Balance of hemostasis. Coagulation and fibrinolytic systems are in perfect balance as long as positive and negative influences are equal. Platelets and coagulation factors drive the clotting process, while plasmin activators drive the opposing fibrinolytic system. Both systems are held in check by regulators and inhibitors.

- If the PT, APTT, and TT are greatly prolonged, the possibility of the presence of heparin should be considered before investigating a factor deficiency.

- The unconfirmed presence of heparin in the patient’s plasma can be confirmed by the addition of protamine sulfate to the plasma sample to inhibit the action of heparin and normalize all prolonged tests.

D. Hypercoagulability disorders are distinguished by pathologic thrombosis in the coronary circulation, cerebral circulation, or the deep veins of the legs.

1. Hypercoagulability resulting from hereditary deficiency is seen mainly in patients younger than 40 years and is often fatal. The most common deficiencies are in antithrombin III, PC, and plasminogen.
   a. Genetically induced deficiencies of natural inhibitors, including PS, PC, and antithrombin III, are found in 25% of persons with this disorder.
   b. Disorders of the fibrinolytic system comprise 30% of reported genetic deficiencies and result from:
      - Decrease in plasminogen or defective function
      - Decrease in tissue-plasminogen activator (t-PA)
      - Abnormal fibrinogen molecule
      - Factor XII/prekallikrein deficiency
   c. Miscellaneous causes of genetic hypercoagulability involve blood vessel and platelet damage.

2. Acquired disorders of hypercoagulability are usually seen in patients older than 40 years and usually occur secondary to a primary pathology, such as lupus anticoagulant, malignancies, or pregnancy.

3. Regulation of systems of hemostasis is kept in balance by inhibitors of coagulation and fibrinolysis (Figure 2–6).

4. Antithrombin III deficiencies affect 1 in 5,000 people.
   a. Inherited deficiencies can be classified as either a Type I or Type II deficiency.
      - A Type I deficiency is characterized by a decrease in activity and a decrease in the antithrombin III molecule.
      - A Type II deficiency is characterized by a decrease in activity, but a normal amount of antithrombin III molecule.
   b. Acquired antithrombin III deficiencies result from the following primary disorders:
      - DIC, which results in consumption of the molecule
      - Cirrhosis, which results in decreased production
      - Nephrotic syndrome, which results from a loss of the molecule in the urine.
      - Medications (e.g., heparin, estrogen, l-asparaginase)

5. PC deficiencies (Figure 2–7) are one of the most common causes of hypercoagulability disorders and can be either inherited or acquired.
Inhibition of coagulation by protein S (PS) and protein C (PC). PS serves as a cofactor in PC inactivation of coagulation factors V and VIII. PS exists as an inactive protein-bound (C4b-Bp) form and an active nonbound form (PSA). PC is activated by thrombin, and activated PC (AcPC) combines with PSA on the platelet surface to inactivate factors V and VIII.

a. **Inherited deficiencies** are genetically heterozygous in 6% to 12% of cases. The majority of PC deficiencies are severe (homozygous). Patients exhibit low or absent PC levels. Low levels result in serious clinical symptoms, such as ecchymotic areas of skin with purpura and tissue necrosis, blindness, and CNS thrombosis.

b. **Acquired deficiencies** are caused by chronic oral anticoagulant therapy with coumarin (vitamin K blocker). The result is coumarin necrosis, which is caused by a rapid functional decrease of PC.

6. **PS deficiencies** can be inherited or acquired. PS is a vitamin K-dependent glycoprotein that is synthesized in the liver.

a. PS circulates in two forms: an inactive form bound to a protein known as C4b-BP, and as freely circulating PS with full functional activity. The active form of PS functions as a cofactor for PC inactivation of factors V and VIII (Figure 2–7).

b. Inherited deficiencies of PS comprise 8% to 11% of the thrombotic population and have been classified into three clinical subtypes (Table 2–6).

c. **Acquired PS deficiencies** can occur secondary to the following conditions:
   1. Oral couagulants (e.g., Coumarin)
   2. Pregnancy
   3. Oral contraceptives
   4. Low vitamin K levels
   5. Liver disease
   6. Acute inflammation
   7. Newborn

7. **Factor V Leiden** is a genetic resistance to activated protein C (APC).

a. Discovered several years ago when an individual with a family history of deep vein thrombosis (DVT) did not show the expected prolongation of his APTT when APC was added.

   1. The disorder was identified as an amino acid substitution in the factor V molecule resulting in the arginine at the PC cleavage site being replaced by glutamine.

   2. The result is that APC cannot bind to factor V to inhibit and, therefore, chronic DVT results.

<table>
<thead>
<tr>
<th>Subtype</th>
<th>Free Protein S (Nonbound)</th>
<th>Functional Activity</th>
<th>Total Protein S</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type I</td>
<td>Low</td>
<td>Low</td>
<td>Low</td>
</tr>
<tr>
<td>Type IIa</td>
<td>Low</td>
<td>Low</td>
<td>Normal</td>
</tr>
<tr>
<td>Type IIb</td>
<td>Normal</td>
<td>Low</td>
<td>Normal</td>
</tr>
</tbody>
</table>
b. Prevalence:
(1) Approximately 5% of general population
(2) Approximately 20% of all DVT cases
(3) Approximately 50% of cases with a family history of DVT
(4) Approximately 60% of pregnancy associated with thrombosis

c. Laboratory Screening:
(1) Resistance to APC prolongation of the APTT has a sensitivity of 90% in detecting the disorder
(2) PCR identification of the abnormal GAGG sequence

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CHAPTER 3
Routine Hematology

JOEL HUBBARD, PhD, MT (ASCP)

I. LABORATORY ANALYSIS

A. Electronic cell counting. Because of manufacturer diversity and the complexity (i.e., performance characteristics) of cell counters that exists among laboratories, individual laboratory instrumentation is not presented here.

1. Multiparameter cell counters provide 10 or more common parameters, including count of red blood cells (RBCs), white blood cell (WBCs), and platelets with a totally automatic diluting system. The most common parameters provided by current cell counters include:
   a. RBC count
   b. WBC count
   c. Hemoglobin (Hb)
   d. Hematocrit (HCT)
   e. Mean corpuscular volume (MCV)
   f. Mean corpuscular hemoglobin (MCH)
   g. Mean corpuscular hemoglobin concentration (MCHC)
   h. Platelet count
   i. Mean platelet volume (MPV) is determined from the platelet histogram curve. The reference range is 6.5 to 12 fl.
   j. Red cell distribution width (RDW) provides an estimate of RBC anisocytosis (size variation).

   (1) Calculation. The RDW (Figure 3–1) is calculated from the following formula:

   \[
   \text{RDW} = \frac{(A - B)}{(A + B) \times k}
   \]

   where \(A\) = the MCV, in which 20% of the RBCs are larger than the rest; \(B\) = the MCV, in which 80% of the RBCs are larger than the rest; and \(k\) = a constant that represents the number that is required to give a normal value of 10.

   (2) The normal reference range for the RDW is 8.5 to 14.5. Samples showing values >14.5 should be carefully checked for anisocytosis.

   (3) Diagnostic use. When considered with the MCV, the RDW can be of use diagnostically, as illustrated in Table 3–1.

2. Electrical impedance
   a. Particles (most often cells) are forced to flow through small openings (i.e., apertures) between two electrodes in an ionic solution.
Normal red cell distribution curve from a RBC histogram. MCV = mean corpuscular volume; RDW = red blood cell distribution width.

(1) Electrical resistance (R). As each cell passes through the opening, the electrical resistance between the two electrodes increases, because cells are poor conductors of electricity.

(2) Voltage (V). As R increases, V increases. A voltage pulse of short duration is produced for each cell that passes through the aperture. The magnitude of voltage is proportional to the cell volume or size, and the number of voltage pulses is proportional to the frequency of particles passing through the aperture.

b. Methods

(1) RBCs and WBCs are counted in duplicate or triplicate. Each of the duplicated counts must agree within a standardized range of deviation from each other.

(2) The MCV is often determined directly from the voltage-pulse heights from the RBC count or histogram curve (see Section I B).

(3) The Hb of the sample is obtained spectrophotometrically from the WBC dilution.

(4) Platelets are counted in duplicate or triplicate in the RBC aperture bath. Particles ranging from 2 to 20 fL [1 femtoliter (fL) = 10^{-15} L = 1 cubic micrometer (mm^3)] in the RBC bath are sorted as platelets and plotted as a platelet histogram.

(5) RBC indices are computed parameters commonly obtained from automated cell counters.

(a) The HCT is computed from the RBC count and the MCV and calculated from the following formula:

\[
\text{HCT\%} = \frac{\text{RBC} \times \text{MCV}}{10}
\]

Table 3–1 Diagnostic Use of Red Cell Distribution Width (RDW) and Mean Corpuscular Volume (MCV)

<table>
<thead>
<tr>
<th>MCV</th>
<th>RDW</th>
<th>Clinical Importance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>Normal</td>
<td>Acute bleeding, anemia of chronic disorders, RBC molecular deficiencies</td>
</tr>
<tr>
<td>Normal</td>
<td>High</td>
<td>Early stages of a nutritional deficiency (including iron deficiency anemias, myelofibrosis, sideroblastic anemia, cytotoxic chemotherapy)</td>
</tr>
<tr>
<td>Low</td>
<td>Normal</td>
<td>Thalassemia minor, anemia of chronic disorders</td>
</tr>
<tr>
<td>Low</td>
<td>High</td>
<td>Iron deficiency anemia, hemoglobinopathies, thalassemia major</td>
</tr>
<tr>
<td>High</td>
<td>Normal</td>
<td>Aplastic anemia</td>
</tr>
<tr>
<td>High</td>
<td>High</td>
<td>Autoimmune hemolytic anemia, folate or vitamin B_{12} deficiency</td>
</tr>
</tbody>
</table>

RBC = red blood cell
(b) The MCH is computed from the MCV and the MCHC and calculated from the following formula (note that 1 μg = 1 picogram [pg]):

\[
\text{MCH pg} = \left(\frac{\text{MCV} \times \text{MCHC}}{100}\right)
\]

or

\[
\text{MCH} = \left(\frac{\text{Hb} \times 10}{\text{RBC count}}\right)
\]

c. Common errors

(1) Missing parameter(s)

(a) Any WBC or RBC count grossly out of the normal range must be treated with suspicion, and the sample must be repeated.

(b) The “rule of three” (i.e., three times the Hb value should agree ± 3% with the HCT value). If it does not, the sample must be repeated.

(c) Any one of the indices being “slightly” out of range must be considered suspicious.

(2) Carryover from high to low WBC counts is a problem with some cell counters because it can amount to a 2% to 3% error. Carryover results from incomplete removal of all WBCs from the counting chamber between counts.

(a) If the ratio of successive counts exceeds 3:1, then the second count may be in error by as much as 5%.

(b) It may be necessary to repeat any low WBC count that follows a high count.

(3) Increased WBC counts >50,000/mm³ may produce a proportional elevation in Hb values because of increased cellular turbidity in the WBC counting baths. In addition, the MCV and HCT may also be increased because the number of WBCs is high enough to produce error when sized and counted in the RBC aperture bath.

(4) Extremely microcytic MCVs may be overestimated by the instrument.

(5) High patient glucose concentrations >400 mg/dL result in intracellular hypopsmolality in RBCs and may cause a high MCV and HCT, with a low MCHC.

(6) Cold agglutinins in high titer give a falsely high MCV, low RBC counts, and very high MCHCs.

(a) A good clue for this problem is to look for RBC clumping in a thin area on the blood smear.

(b) Warming the blood or diluent to 37 °C may eliminate this problem.

(7) Very high plasma lipid levels resulting in lipemic plasma may produce turbidity in the WBC aperture bath and falsely increase the Hb, MCH, and MCHC.

B. Blood cell histograms are provided by many high-volume instruments to provide size distributions of the different cell populations. The volume, given in mm³ or fl, is plotted against the relative frequency for platelets, WBCs, and RBCs (Figure 3–2).

1. The WBC histogram provides a count and plot of cells in the WBC aperture bath larger than 45 fl.

a. Normal WBC histograms have three distribution peaks:

(1) The first peak, ranging from 45 to 90 fl, represents a small mononuclear population of cells (i.e., lymphocytes).

(2) The second peak, ranging from 90 to 160 fl, represents a minor population of larger mononuclear cells (i.e., monocytes). An increase in the number of cells in this size range can also represent abnormal cell types, such as the immature precursor cell types found in patients with leukemia.
Figure 3–2 Typical cell histogram of normal blood. Note the normal distribution curves of the WBC, RBC, and platelet (PLT) cell populations. The histogram was created by a Coulter model S-Plus IV cell counter. GR = granulocyte; HCT = hematocrit; HGB = hemoglobin; LY = lymphocyte; MCH = mean corpuscular hemoglobin; MCHC = mean corpuscular hemoglobin count; MCV = mean cell volume; MO = monocyte; MPV = mean platelet volume; RDW = red cell distribution width.

(3) The major population and third peak, which ranges from 160 to 450 fL, represents normal mature types of granulocytes.

b. Calculated values
(1) A percentage value for granulocytes, lymphocytes, and monocytes is calculated combining the distribution values and the total WBC distribution spread. Newer cell counters also give values for eosinophils and basophils.
(2) An absolute value of each cell fraction (in cells/mm³) is calculated from the product of the total WBC count and the percent fraction of each cell type.

c. Abnormal WBC histogram patterns can alert the technologist and physician to possible pathology and alert the technologist to include a manual differential count.
(1) The lower threshold is 45 fL, but the histogram will extend lower to detect abnormalities, as shown in Figure 3–3.
(2) Error flags
(a) Region code (R) flags signal irregularities in the WBC distribution and will appear next to the differential parameters that are in error. The “R” stands for the region. The following numerals indicate the location in the WBC histogram where the interference was detected:
   (i) R1 warns of increased interference in the area left of the lymphocyte peak (approximately 35 fL), which is typically caused by sickled RBCs, nucleated RBCs, or clumped and giant platelets being counted in the WBC aperture bath (see Figure 3–3).
CHAPTER 3 Routine Hematology

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(ii) R2 warns of excessive overlap of cell populations at the lymphocyte/mononuclear cell boundary (approximately 90 fL) caused by the presence of abnormal cell types, such as atypical lymph, blast, or plasma cells (Figure 3–4).

(iii) An R3 warning (Figure 3–5) is caused by excessive overlap of cell populations at the mononuclear/granulocyte boundary (approximately 160 fL), which is due to the increased presence of immature granulocytes (i.e., bands, metamyelocytes).

(iv) An R4 warning (Figure 3–6) is caused by the extension of the cell distribution past the upper end of the WBC threshold (approximately 450 fL). This most commonly occurs when the granulocyte population is very high.

(v) RM is the error code for multiple region overlap.

(b) Other signal flags include H, which occurs when a parameter value is higher than the set normal limit, and L, which occurs when a parameter value is lower than the set normal limit.

2. RBC histograms represent the cells counted in the RBC dilution, in the size range of 36 to 360 fL, which are sorted and plotted as frequency against cellular volume.

a. Normal RBC histogram. A single peak should be found normally between 70 fL and 110 fL, and the peak should coincide with the MCV (Figure 3–7).

b. Abnormal RBC histograms result when the MCV of the curve falls outside of the normal range of 80 to 100 fL or when the RDW is > 14.5.

(1) An increased MCV shifts the curve to the right, and a decreased MCV shifts the curve to the left.

(2) An increased RDW is reflected by an increase in the "width" of the area beneath the curve (Figure 3–8).

(3) In some disorders, there may be two populations of RBCs (i.e., a microcytic population and a macrocytic population of cells (Figure 3–8)).

![Figure 3–3 Abnormal WBC histogram representing an R1 distribution. Note the high takeoff from the Y axis. GR = granulocyte; LY = lymphocyte; MO = monocyte.]

![Figure 3–4 Abnormal WBC histogram representing an R2 distribution. GR = granulocyte; LY = lymphocyte; MO = monocyte.]
3. Platelet histograms represent cells in the RBC counting baths that are in the size range of 2 to 20 fL. Cells in this range are counted, and their frequency is plotted against cellular volume. Atypical platelet histograms can result in some disorders when large platelets are present (Figure 3–2).

C. X-Bar-B (\( \bar{X}_B \)) statistical analysis

1. Background. Research has shown that RBC indices (i.e., MCV, MCH, MCHC) of patient populations are stable over time. This stability is the basis of a hematology quality control technique known as \( \bar{X}_B \) analysis.

2. Function. Use of this statistical tool quickly determines the direction and amount of daily change caused by the instrument, reagents, or sample handling.

3. Establishment
   a. Target values of MCV, MCH, and MCHC are determined by calculating the mean of 250 to 1,000 samples for each of the three parameters.
   b. After target values are determined, ongoing analysis can be applied using small "batches" of 20 samples.
   c. The mean and standard deviation of each batch is compared with the target value of each parameter and averaged with the mean and standard deviation of the previous batches.

4. The hematology system is considered “in control” when the batch means are within established standard deviation limits of the target values.

5. The percent difference between each batch mean and its corresponding target value can be calculated and displayed on a Levy-Jennings graph.
CHAPTER 3 Routine Hematology

D. Laser scatter counting and flow cytometry

1. A wide range of diagnostic applications can be found from using the principles of laser scatter and flow cytometry.
   a. Routine cell counting and differential separation of the white cells can be routinely performed.
   b. These techniques can be used to help presumptively characterize acute and chronic leukemias and lymphomas.
   c. Specialized flow cytometry instrumentation can differentially separate types of leukemic cells, tumor cells, or subtype lymphocyte functional types (e.g., B lymphocytes, T lymphocytes, T-lymphocyte subtypes).
   d. These types of cell counters can be adapted to perform reticulocyte counts.
   e. One of the most far-reaching applications of flow cytometry technology is the DNA analysis of rapidly growing tumor tissue.

2. Principles of operation combine chemistry and flow cytometry for the evaluation of individual blood cell populations in each of several flow cells or “channels.”
   a. General steps of flow cytometry include:
      (1) Preparation and staining of cell populations with cytochemical marking for further analysis
      (2) Flow cell measures of cell size, cytochemical staining properties, and frequency of each cell type
      (3) Computer conversion of measurements into common hematologic parameters
   b. In the RBC channel, RBCs are diluted and passed through a flow cell for counting with the technology of laser scatter.
      (1) RBC indices and RDW are computed and reported from the total RBC count.
      (2) Many flow cytometry instruments provide RBC morphology measurements.
   c. The WBC/myeloperoxidase (MPO) channel consists of a specialized flow cell in which leukocytes are counted and differentiated.
      (1) The blood is diluted, the RBCs are lysed, and the WBCs are stained for MPO activity. MPO is found in the greatest amount in granulocytes.
Slight-to-moderate amounts of MPO exist in monocytes; very little exists in lymphocytes and immature leukocyte precursor cells (i.e., blasts).

(2) The WBCs enter a flow cell where two-dimensional light-scatter and light-absorption properties are determined for each cell.

(3) Stained cells absorb more light and scatter light at a different angle than unstained cells. The end result is a two-dimensional leukocyte cytogram.

(4) The peroxidase (MPO) channel gives a total WBC count and absolute numbers of neutrophils, lymphocytes, monocytes, and large unstained cells (LUC). The mean peroxidase activity is expressed as the mean peroxidase index (MPXI) and scaled from $-10$ to $+10$.

d. Basophils. In some flow cytometry instruments, basophils are not classified in the peroxidase channel because they appear in the same area on the scattergram as lymphocytes. Basophils, therefore, require separate analysis in a separate channel.

(1) RBCs are fully lysed, and WBCs are partially lysed, leaving only anucleated leukocytes.

(2) Cells in this channel enter a flow cell and are analyzed by two-angle light scatter from a laser source.

(3) Basophils are more resistant to lysis than other leukocytes. They are counted and sorted separately.

(4) Due to variations in nuclear texture, segmented nuclei have a higher component of wide-angle scatter.

(5) Based on their properties of wide-angle light scatter (Figure 3–9), a ratio of segmented nuclei to nonsegmented nuclei (i.e., immature myeloid cells) is reported as a lobularity index (LI).

(a) A high LI indicates a large population of segmented nuclei

(b) A low LI indicates a morphologic shift to the left, with more bands and immature neutrophil forms.

3. Cytoflow principle of operation

a. Flow cytometry is the automated analysis of cells and other particles passing in a fluid suspension through a laser light source. Cells are first labeled with monoclonal antibodies specific for a variety of cell membrane protein receptors.

(1) Monoclonal antibodies are commercially available and provide highly specific for identification of cell maturation.

(2) These reagents are specific enough to recognize a single antigenic site (epitope) of not more than 5 to 7 amino acids on a cell.

(3) Can be used in the identification of lymphocyte subpopulations and maturation stages of granulocytes and monocytes.

(4) These monoclonal markers are commonly known as cluster designation (CD) markers.

(5) The CD designation can define both cell types and levels of maturation within a cell line. Presently, there are over 100 commercially available CD markers available.

(6) By using a panel of antibodies that covers a spectrum of CDs, a heterogeneous cell population such as the bone marrow or a lymph node can be accurately characterized and quantitated.

b. To be distinguished and differentiated in flow analysis, CD markers must first be tagged with a fluorochrome or chromophore, to be able to absorb the light the laser emits.

(1) The tagged cell emits light back at a wavelength sufficiently longer than the excitation light so that the two wavelengths can be optically separated with selective filters.

(2) Fluorescein isothiocyanate (FITC) is the most common fluorochrome used in cytflow. If multiple fluorochromes are used to identify more than one cell population, their emission spectra must have minimal overlap so they can be separated and quantitated.

c. Fluorochrome tagged cells are channeled in a fluid stream to pass single file through a beam of laser light.
Figure 3–9 Typical format of a scattergram cell distribution report of a flow cytometer cell-counting instrument. Large unstained cells (Luc), basophils (Baso), eosinophils (Eos), neutrophils (Neut), and monocytes (Mono) are represented.

Lasers emit monochromatic light, or light of a single wavelength. As each cell passes through and breaks the laser beam, photons of light are scattered and emitted by the cells to be separated into the resulting wavelengths by a series of filters and mirrors known as a photomultiplier tube. The separated light then is passed to individual detectors that will generate electrical impulses proportional to the amount of light striking the detector. The intrinsic cellular characteristics of each cell can be analyzed by the angles of light scatter. Intracellular characteristics are detected by forward-angle light scatter (FALS) and by side scatter (SS).

(a) FALS is related to the light collected by the photomultiplier tube along the axis of the laser, and is proportional to cell size as shown in Figure 3–10.

(b) The light deflected off the cells at a 90 degree angle to the laser beam is SS. The reflected light reveals information about cell density, nuclear complexity, and cell granularity.

(c) Deflected light is converted to a scattergram plotting FALS on the y-axis against SS on the x-axis. Each dot will represent a single cell of given size and density. Emitted light from tagged fluorochromes is converted to a histogram which plots increasing intensity of cellular fluorescence on the x-axis against the cell frequency on the y-axis (Figure 3–11).
Figure 3–10 Cytoflow scattergram example of white cell differentiation by 90 degree angle light scatter on the x-axis compared to the amount of forward angle light scatter on the y-axis.

II. HEMATOPOIETIC TISSUES are organs and tissue areas in which blood cell production or regulation occurs. These tissues include areas of fetal hematopoiesis, the spleen, lymphatic tissues, and the bone marrow.

A. Embryonic and fetal hematopoiesis
1. Primitive erythroblasts are the first blood cells formed by the first month of embryonic life. These cells are formed outside the embryo in the mesenchyme of the yolk sac.
2. By the sixth week of embryonic life, the liver becomes the primary hematopoietic organ for producing definitive erythroblasts, which mature to non-nucleated RBCs.
3. In mid-fetal life, the spleen and lymph nodes begin a limited role as secondary lymphoid organs.
4. In the last half of fetal life, bone marrow hematopoiesis begins and becomes progressively more important. Hematopoiesis in the liver begins to slowly diminish by the last trimester.
5. Shortly after birth, hematopoiesis ceases in the liver, and the marrow becomes the only site for production of erythrocytes, granulocytes, monocytes, platelets, and B lymphocytes.
   a. Hematopoietic primitive stem cells and committed progenitor cells are located in the marrow.

Figure 3–11 Fluorescence scattergram demonstrating the amount of CD4 positive cells by plotting the wavelength of fluorescence on the x-axis compared to the frequency on positive cells on the y-axis. The scattergram shows a prominence of CD4 lymphocytes in the cell population analyzed.
b. In an infant, most bone marrow is actively hematopoietic.

c. With increasing age, marrow for hematopoiesis becomes progressively limited.

d. The spleen and lymph nodes serve as secondary lymphoid tissue for lymphocyte development and differentiation.

B. Spleen

1. Internal structure is divided into white pulp and red pulp.
   a. The pulp is divided by fibrous trabeculae, which contain:
      (1) Arteries that emerge from the trabeculae, giving off right-angle branches into the white pulp and terminating in lymphatic nodules
      (2) Veins that drain the venous sinuses of the red pulp into the hepatic-portal blood vessels
      (3) Lymphatics that are scattered throughout the spleen and pass through lymphatic nodules
   b. White pulp is so named because it contains concentrations of WBCs (i.e., lymphocytes and macrophages) in anatomically select areas of the spleen.
      (1) A periradial lymphatic sheath, consisting of T lymphocytes, is wrapped around the central artery.
      (2) Lymphatic nodules, located throughout the white pulp, contain a germinal center.
         (a) The marginal zone, which surrounds the germinal centers and separates the red and white pulp, is rich in T lymphocytes and macrophages.
         (b) The nodules are held together by a network of fibrous reticular cells.
   c. Red pulp is so named because it contains venous sinuses and cords of fibrous trabeculae.
      (1) Blood from marginal zones and central arterial terminals in the white pulp drains into the red pulp.
      (2) RBCs that enter the cords must pass through a porous membrane separating the cords from venous sinuses.
         (a) Pores in the fibrous trabeculae are only approximately 3 μm in diameter.
         (b) RBCs, which are an average of 7 μm, must be squeezed through the small pores.
         (c) RBCs that are old or contain cytoplasmic inclusions do not have the necessary pliability to transverse the pores, and they are destroyed.

2. Functions of the spleen
   a. RBC filtration occurs to eliminate cellular impurities.
      (1) The process of “squeezing” RBCs through the narrow fenestrated cord selectively eliminates old or abnormal RBCs.
      (2) RBCs and platelets coated with autoimmune gamma G immunoglobulin are also destroyed by the macrophages in the red pulp.
   b. Cell grooming or restructuring of RBCs with intracellular inclusions occurs in the red pulp.
      (1) Reticulocytes are delayed in transit because of small amounts of cytoplasmic RNA.
      (2) RBC cytoplasmic inclusions such as deoxyribonucleic acid (DNA), ribonucleic acid (RNA), iron, denatured Hb, or a remaining nucleus are squeezed from RBCs as they work their way through the tiny pores. Cellular debris is eliminated by splenic macrophages.
   c. In humans, the spleen serves as a reservoir for platelets and lymphocytes.
      (1) Up to 30% of circulating platelets are sequestered by the spleen through slow transit.
      (2) The spleen is rich in mature lymphocytes destined for antigen-dependent differentiation.
   d. The spleen serves as an important organ involved in immunity.
      (1) White pulp contains approximately 25% of the peripheral T lymphocytes in transit between circulation and tissue.
Approximately 10% to 15% of peripheral B lymphocytes are found in splenic nodules.

The spleen provides a locale for immunity-induced lymphocyte differentiation and antibody production. It serves as an important organ for immunoglobulin M (IgM) production by B lymphocytes.

Asplenia (the absence of splenic function) can be caused by either surgical removal (e.g., splenectomy) or radiation overexposure. This condition can also occur in association with malabsorption syndromes. The hematologic results of asplenia include:

- Increased susceptibility to infection
- Acute granulocytosis
- Acute thrombocytosis, with occasional giant platelets
- Chronic and absolute lymphocytosis and monocytosis
- Increased appearance of immature RBCs in the circulation
- Increased amount of circulating RBCs with cytoplasmic inclusions or abnormal forms (e.g., Howell-Jolly bodies, target cells, and burr cells)

Splenomegaly

- Definitions
  1. Splenomegaly describes an enlarged spleen.
  2. Hypersplenism describes exaggerated inhibitory or destructive functions of the spleen, which is usually accompanied by splenomegaly.

- Clinical presentation. Splenomegaly results in vascular congestion and portal hypertension. RBCs, granulocytes, and as many as 90% of circulating platelets may be trapped, which results in anemia, leukocytopenia, and thrombocytopenia. A hypercellular marrow results in response to a chronic pancytopenia.

Lymphatic tissues

1. Lymphopoiesis is anatomically divided between two areas.
   - Primary lymphatic tissues include the bone marrow for B lymphocytes, the thymus for T lymphocytes, and sites of active hematopoiesis in the fetus.
   - Secondary lymphatic tissues serve as reservoirs for already differentiated lymphocytes. These tissues include the lymph nodes, spleen, and gut-associated lymphatic tissue.

2. The thymus is a primary tissue for T-lymphocyte development.
   - The cortex, or outer part of the organ, consists of several cell types.
     1. Small and medium primitive T cells (i.e., thymocytes) are the youngest cells found in the thymus.
     2. Epithelial cells serve as important effectors of T-cell differentiation.
     3. The corticomedullary junction is rich in macrophages.
   - The medulla, or interior of the organ, is rich in maturing T lymphocytes, epithelial cells, and concentric swirls of squamous epithelial cells known as Hassall's corpuscles. Mature immunocompetent T lymphocytes leave the medulla and enter the circulation to migrate to secondary lymphoid tissues to await “specific” immune differentiation.

3. Lymph nodes, spread throughout the body in a “second” vascular system, comprise part of the secondary lymphatic system.
   - All lymph empties and is recirculated into the bloodstream via the right thoracic duct and the left thoracic duct, which is the major lymphatic vessel.
   - All nodes are separated from each other by thin-walled vessels known as lymph ducts. The normal anatomic structure of each node includes a cortex, paraaortic, and medulla.
     1. The cortex contains lymphocyte follicles (nodules) with germinal centers. These follicles are unique to lymph nodes and the spleen.
       1. All follicles are arranged in a row beneath the surface capsular lining.
       2. These follicles contain a concentration of macrophages and B lymphocytes.
       3. The germinal centers are surrounded by a high concentration of T lymphocytes.
The paracortex is defined as the area between the cortex and medulla. This area of the node is rich in macrophages, as well as B and T lymphocytes in transit. The medulla, or inside of the node, is arranged in parallel cords of small lymph and plasma cells.

4. Lymphopoiesis is defined as lymphocyte production or regulation in primary or secondary lymphatic tissues.
   a. Lymphocyte production in primary tissues is continuous and independent of antigen stimulation.
      1. The production rate is in excess of demand.
      2. Only a fraction of newly formed lymphocytes in the primary tissues survive to gain access to secondary lymphoid organs.
   b. Lymphocyte proliferation in secondary tissues is antigenic stimulation-dependent, resulting in the proliferation of specific clonal populations of lymphocytes to carry out the immunologic response.
   c. Of circulating lymphocytes, 75% to 85% are T lymphocytes, and 10% to 15% are B lymphocytes.
   d. T lymphocytes are more actively motile and recirculate more than B lymphocytes.
      1. T cells migrate among the blood (30 minutes), spleen (6 hours), and lymph nodes (15–20 hours).
      2. B lymphocytes do not freely circulate and may stay in a lymph node for as long as 30 hours.

D. Bone marrow
   1. Structure and development
      a. Fat-cell occupation of the bone marrow space begins by 4 years of age, at which time the growth of bone cavities has exceeded the body’s need, and the available space in bone cavities has grown faster than the needed circulation blood mass.
         1. Fat-cell growth occurs first at the diaphysis of long bones and slowly extends to the center of the bone.
         2. By 18 years of age, fatty replacement has limited active hematopoietic marrow to the vertebrae, ribs, skull, sternum, proximal epiphyses of long bones, and the iliac crest of the pelvis.
         3. Because of this available fatty bone marrow reserve, reactivation of extramedullary organs rarely takes place.
      b. Organization. The marrow is organized in a spoke-like pattern of venous sinuses and cords of hematopoietic tissue.
         1. Venous sinuses are covered on the marrow side by endothelial cells and on the sinus side by a basement membrane and reticular adventitial cells, which put out projections or “nests” to support hematopoietic cells.
         2. Megakaryocytes lie within the cords close to the sinus wall. Strings of platelets peel directly into the venous sinus.
         3. Erythroblasts lie close to venous sinuses in clusters or colonies.
            a. Each cluster consists of a central macrophage (i.e., nurse cell) surrounded by erythroblasts in various stages of maturation.
            b. When mature, the reticulocyte squeezes through the basement membrane and endothelial layer to be released into the venous sinus.
      c. Nerve supply. The marrow has an extensive supply of nerves, which may play an important autoregulatory role to adjust blood flow to the rate of cellular maturation and proliferation.
   2. Bone marrow functions
      a. Minor. The marrow has a minor function in the antigen processing of cellular and humoral immunity.
      b. Major. The major function of the marrow is the production and proliferation of blood cells (hematopoiesis). Marrow hematopoiesis is divided into three major compartments or cell types (Figure 3–12).
Figure 3–12  Hematokinetics in the bone marrow. Hematopoiesis in the marrow consists of three compartments: multipotential stem cells, unipotential stem cells, and precursor cells. Cell types include the following: Colony-forming unit (CFU) in the spleen (CFU-S), lymph (CFU-L), erythroid cells (CFU-E), megakaryocytes (CFU-MEG), and granulocytes/monocytes (CFU-GM); burst-forming unit erythroid (BFU-E).

1. Stem cells, known as pluripotential or multipotential cells, retain the ability to differentiate into any cell line.
   (a) The stem cells are referred to as colony-forming units-spleen (CFU-S).
   (b) CFU-S differentiate in either of two pathways, giving rise to either secondary multipotential stem cells, which give rise to primitive B or T lymphocytes, or multipotential stem cells, which give rise to the non-lymphocytes.

2. Progenitor (committed) cells are also known as unipotential stem cells, because they differentiate into only one cell line. Committed stem cells include BFU-E, CFU-E, CFU-MEG, and CFU-GM (see Figure 3–10 for descriptions of abbreviations).

3. Precursor cells comprise the third marrow compartment. Each type of unipotential stem cell matures into a blast form (e.g., myeloblast, megakaryoblast, erythroblast).

3. Bone marrow examination can be performed with an aspirate and a biopsy, both of which are nonsurgically obtained by a pathologist from either the sternum or the pelvic iliac crest.
   a. Background. The weight of the marrow in an adult is 1,300 to 1,500 g. Marrow can undergo complete transformation within hours to days. A marrow examination is vital to the diagnosis of many diseases, such as myeloproliferative diseases, lymphoproliferative diseases, and some severe anemias of unknown origin.
   b. Preparation of aspirate. A marrow aspirate must be prepared with speed and quick drying to prevent clotting of the specimen. The types of preparations made with an aspirate include:
      (1) Marrow films stained with Wright’s stain
      (2) Direct films stained with Wright’s stain
      (3) Marrow imprint stained with Wright’s stain
      (4) Crush preparations stained with Wright’s stain
      (5) Histologic study of marrow particles
      (6) Gross quantitative study of marrow
c. Marrow preparations from aspiration or biopsy are stained for cell morphology or iron content.
   (1) The Wright’s stain is most commonly used.
   (2) Prussian blue stain is used for the quantization of iron in the macrophages of the marrow.
      (a) Hemosiderin or ferritin are stained, and the staining intensity is graded from 1+ to 4+ (2+–4+ is normal for adults).
      (b) Sideroblasts (i.e., normoblasts containing one or more particles of stainable iron) can be examined and quantitated.
         (i) Normally, 20% to 60% of the late normoblasts are sideroblasts.
         (ii) Sideroblasts are decreased with various iron storage anemias.
   d. Examination of cellularity is expressed as the ratio of the volume of hematopoietic cells to the volume of marrow space for the patient’s age.
      (1) A current peripheral blood cell count, platelet count, and reticulocyte count [see Section IV F 5 c (2)] should be included with a marrow examination.
      (2) Cellularity is estimated from gross quantitative study and histologic sections of marrow biopsies.
      (3) Irregularities of cellular distribution are first examined with a low-power scan of the slide, then examined at 500× or 1,000× for cellular characteristics.
         (a) A differential count of 300 to 1,000 cells is completed.
         (b) A myeloid/erythroid (M:E) ratio is estimated by comparing the percent of total granulocytes with total normoblasts. The normal M:E ratio in the adult is 2:1 to 4:1.

III. HEMOGLOBIN SYNTHESIS, STRUCTURE, AND FUNCTION

A. Heme synthesis (Figure 3–13)
   1. Synthesis occurs on the mitochondria of normoblasts and begins with succinyl-coenzyme A (SCA), which is a by-product of the tricarboxylic acid (TCA) cycle.
a. SCA combines with glycine to yield an unstable intermediate known as \( \alpha \)-amino-\( \beta \)-ketoadipate.

b. The intermediate is decarboxylated to form delta (\( \delta \))-aminolevulinic acid (ALA).
   1. This reaction occurs in mitochondria and requires pyridoxal phosphate (i.e., vitamin B\(_6\)).
   2. Trace amounts of ALA, which is normally found in urine, are increased in certain abnormalities of heme synthesis (e.g., lead poisoning).

c. Two molecules of ALA combine to form porphobilinogen (PBG).
   1. Normally, trace amounts of PBG can be measured in urine.
   2. Increased amounts of PBG are excreted in acute intermittent porphyria and are detected by a color reaction with Ehrlich's aldehyde reagent.

2. Four molecules of porphobilinogen combine to form uroporphyrinogen I or III.
   a. The type III isomer form is converted by way of coproporphyrinogen III, and protoporphyrinogen IX to protoporphyrin IX.
   b. Iron is inserted into protoporphyrin by the mitochondrial enzyme, ferrochelatase, to complete the formation of the heme moiety.
   c. In certain diseases, this pathway may be partially blocked.
      1. Type I isomers of uroporphyrinogen and coproporphyrinogen are formed and excreted in excess urinary amounts as uroporphyrin I and coproporphyrin I.
      2. Protoporphyrin is normally found in mature RBCs, but concentrations are increased in lead poisoning and iron deficiency anemia.

B. Globin chain synthesis
1. Polypeptide chains are manufactured on ribosomes in the normoblast cytoplasm.
2. Globin chains are assembled from two pairs of polypeptide chains (i.e., four chains per hemoglobin molecule).
3. Four primary chains (i.e., \( \alpha \), \( \beta \), \( \gamma \), \( \delta \)) can be produced. Each type of globin chain is different by only a few amino-acid substitutions.
4. There are many Hb forms, depending on the combination of the two pairs of globin chains.
   a. An embryonic form, \( \alpha_2 \varepsilon_2 \), is detected early in fetal life.
   b. By 3 months of embryonic life, embryonic Hb is replaced by fetal Hb (Hb F).
      1. Hb F consists of two \( \alpha \) chains and two \( \gamma \) chains (i.e., \( \alpha_2 \gamma_2 \)).
      2. Hb F is the major Hb in the fetus and newborn infant.
      3. Hb F has a higher oxygen affinity than that of adult Hb.
      4. \( \beta \)-chain production does not begin until the 20th week of prenatal life, so adult Hb is approximately 10% between 20 and 35 weeks, and 15% to 40% at birth.
      5. After birth, the production of Hb F slowly ceases, and by 6 months of age, it constitutes <8% of the total Hb content.
   (a) By 1 year of age, infants have <2% Hb F.
   (b) Less than 1% Hb F is normally found in adults. Reactivation of Hb F production may occur in pregnancy and in some disorders of erythroblastosis.
   c. Adult hemoglobin (HbA) is the major adult form, consisting of two \( \alpha \) chains and two \( \beta \) chains (i.e., \( \alpha_2 \beta_2 \)).
   d. Hemoglobin \( \alpha_2 \) (HbA\(_2\)) accounts for 1.5% to 3.5% of normal adult hemoglobin.
      1. HbA\(_2\) consists of two \( \alpha \) chains and two \( \delta \) chains (i.e., \( \alpha_2 \delta_2 \)).
      2. \( \delta \)-Chain synthesis occurs only in normoblasts and is absent in reticulocytes.
   (3) The HbA\(_2\) form is increased in some \( \beta \)-thalassemias and in iron deficiency anemia.
   e. Genetic control of globin-chain production is seen as gene separation.
      1. The production of \( \alpha \)-chains is coded on chromosome 16.
      2. The production of all other globin chains is coded on chromosome 11.

C. Structure and function of hemoglobin
1. The main function of an RBC is to contain, transport, and protect hemoglobin molecules.
2. Each Hb molecule consists of four globin chains (most commonly, α₂β₂) and four heme groups, each with a center iron molecule.

3. Each globin chain has a hydrophobic “pocket” that contains a heme group.
   a. This arrangement protects the Fe²⁺ from oxidation to the ferric form (i.e., Fe³⁺).
   b. The ferric form cannot bind oxygen.

4. The iron of each heme is directly bonded to a nitrogen atom of a histidine side chain. This histidine is known as the proximal histidine and functions to increase the oxygen affinity of the heme ring.

5. A second histidine, known as the distal histidine, is on the opposite side of the heme plane. This histidine sterically diminishes the binding of carbon monoxide (CO) and inhibits the oxidation of the heme iron to the ferric state.

6. One molecule of Hb can bind up to eight atoms of oxygen (i.e., two oxygens per heme ring).

7. Hemoglobin exhibits three kinds of allosteric effects (i.e., interactions that occur between spatially distinct sites within the molecule).
   a. Cooperative binding of oxygen increases the amount of oxygen that can be carried by a hemoglobin molecule.
      (1) The binding of one molecule of oxygen to a heme group facilitates the further binding of oxygen to other heme groups in the same molecule.
      (2) This property is responsible for the pattern seen with the sigmoid-shaped hemoglobin oxygen dissociation curve, as illustrated in Figure 3–14.
   b. The Bohr effect is the chemical phenomenon whereby protons (i.e., H⁺ atoms) and carbon dioxide (CO₂) promote the release of oxygen from the Hb molecule.
      (1) This characteristic is physiologically important to enhance the release of oxygen in metabolically active tissues.
      (2) Each RBC contains an enzyme known as carbonic anhydrase (CA), which catalyzes the conversion of the CO₂ given up by tissues and water (H₂O) to produce carbonic acid (H₂CO₃), as shown by the chemical equation that follows:

      \[ \text{Metabolically active tissue} \quad \text{CA} \rightarrow \text{CO}_2 + \text{H}_2\text{O} \rightarrow \text{HCO}_3^- + \text{H}^+ \]

      (3) The H⁺ liberated by this reaction binds to sites on the globin chain, lowering the Hb oxygen affinity and releasing more oxygen to the tissues.
      (4) A small portion of the CO₂ binds to amino-end terminal groups of the globin chains and is transported to the lungs as carboxy-hemoglobin. The binding of CO₂ further lowers Hb oxygen affinity.
      (5) In the lungs, the binding of oxygen releases H⁺ and displaces bound Hb-CO₂, as illustrated by the chemical reaction that follows:

      \[ \text{HCO}_3^- + \text{H}^+ \rightarrow \text{CO}_2 + \text{H}_2\text{O} \]
c. The third allosteric effect demonstrated by Hb is the regulation of the oxygen affinity of Hb by 2,3-diphosphoglycerate (2,3-DPG).
   (1) Only one molecule of 2,3-DPG can be bound per Hb molecule by cross-linking between the two β chains.
   (2) 2,3-DPG binds more weakly to HbF than to HbA, which partially explains why HbF has a higher oxygen affinity.

d. Allosteric properties of Hb arise from interactions between the α and β chains. Hemoglobin can exist in two allosteric forms:
   (1) The T (i.e., tense) form is the low oxygen affinity state of hemoglobin.
       (a) The quaternary structure of Hb is stabilized by noncovalent electrostatic bonds between the different globin chains.
       (b) Globin-chain bonds partially “close off” the heme pockets, making accessibility of oxygen to the heme iron more difficult.
       (c) On oxygenation in the lungs of the first heme, the iron moves into the plane of the heme and pulls the proximal histidine.
       (d) The resulting movement of the histidine breaks some of the noncovalent chain-chain bonds, opening up the reactive sites of the Hb molecule and shifting the equilibrium from the T form to a high-affinity state.
       (e) The T form is stabilized by any one of the following:
           (i) Binding of 2,3-DPG
           (ii) Binding of CO
           (iii) Binding of H⁺
   (2) The R (i.e., reactive) form refers to the high oxygen affinity state, in which the iron in each heme ring is readily accessible to oxygen binding.

D. Oxygen transport
1. Regulation. The amount of oxygen reaching the tissues can be regulated by either altering the number of circulating RBCs, which causes a change in the rate of erythropoiesis, or by altering the affinity of hemoglobin for oxygen.
2. In a normal steady state, the amount of oxygen and CO₂ exchanged in the lungs is equal to the amount exchanged in the tissues.
3. Oxygen is carried in the blood in two forms.
   a. Approximately 3% of oxygen is dissolved in the plasma.
   b. The majority of oxygen is carried by Hb in the RBCs. Each gram of Hb has the maximum capacity to bind 1.34 mL of oxygen.
4. Carbon monoxide binds 210 times stronger than oxygen to a hemoglobin molecule.
   a. If a person were breathing room air (i.e., 21% oxygen) contaminated with as little as 0.1% CO, half of the Hb-binding sites would be filled with CO.
   b. In addition to lowering Hb-oxygen saturation, CO results in a left shift of the oxygen dissociation curve, which reflects an increased Hb-oxygen affinity state.

E. Carbon dioxide transport
1. Approximately 5% of the total CO₂ in arterial blood is physically dissolved in plasma.
2. Approximately 5% of the CO₂, known as carbamino-CO₂, is carried in blood bound to amino groups of plasma proteins.
3. Approximately 90% of the blood CO₂ is converted to bicarbonate and H⁺ ions.

F. Abnormal hemoglobin variants
1. Carboxyhemoglobin (HbCO) is a carbon monoxide (CO) variant of Hb found in the blood at levels <1% of the total Hb in a normal individual.
   a. Pathophysiology
      (1) Hb has a 200 times greater affinity for CO than O₂.
      (2) Very small amounts in the atmosphere lead to asphyxiation.
         (a) As little as 0.04% (v/v) of CO can result in a HbCO blood level of 10%.
(b) Exposure of CO up to a level of 0.1% (v/v) can increase HbCO blood levels to 50% to 70% resulting in:

(i) Unconsciousness
(ii) Respiratory failure
(iii) Death

b. Laboratory diagnosis
(1) A blood level of 0.5% is typical in nonsmokers.
(2) Blood levels of up to 5% are typical of chronic smokers.
(3) Assays consist of screening and quantitative methods.
   (a) Screening methods use a mixture of a patient hemolysate and 1 Mol/L NAOH. Samples with more than 20% HbCO result in a light red end product as compared to a brown end product for a normal sample.
   (b) Quantitative methods consist of gas chromatography and spectrophotometry.

c. Treatment consists of removal of source and hyperbaric O₂ therapy.

2. Sulfhemoglobin is an Hb variant resulting from the oxidative degeneration of Hb by the addition of a sulfur atom to each Hb molecule.

a. Pathophysiology
   (1) The O₂ affinity of sulfhemoglobin is reduced to one hundredth the affinity of normal Hb.
   (2) Is an acquired condition producing cyanosis at levels exceeding 3% to 4% Hb content.
   (3) Results from exposure to certain sulfur-based drugs (i.e., sulfonamides), and chemicals.

b. Laboratory diagnosis may be performed by analysis of a sample hemolysate for an increase in an absorption band at 620 nm.

c. Treatment consists of removal of cause.

3. Methemoglobin (HbM) is Hb with the heme-iron in the ferric (Fe³⁺) valance.

a. Pathophysiology
   (1) Normal Hb is converted to 0.5% to 3.0% of HbM daily which is reduced by RBC metabolic pathways [IV, D].
   (2) Mild clinical symptoms of HbM result from three physiological causes.
      (a) Inherited methemoglobinemia can occur in two forms.
      (i) Is usually due to inheritance of a decrease in the NADH-methemoglobin reductase enzyme.
      (ii) Various amino acid substitutions in the globin chain that directly affect heme groups by shifting the iron to the ferric state sometimes occur. There are five variants.
      (b) Acquired methemoglobinemia can be caused by a variety of substances.
          (i) Antimalarial drugs
          (ii) Sulfonamides
          (iii) Drugs of abuse
          (iv) Nitrate-rich water or foods

b. Laboratory diagnosis
   (1) Heinz bodies can be demonstrated on a peripheral blood preparation with crystal violet staining.
   (2) Diaphorase screening tests with specific enzyme assays can quantitate levels of HbM.
   (3) Quantitation with spectrophotometry.
      (a) HbM levels above 1.5% of the total Hb demonstrate a characteristic absorbance peak at 630 nm.
      (b) KCN is added causing HbM to convert to cyanmethemoglobin which does not absorb at 630 nm
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Differences in absorbance before and after KCN addition are proportional to HbM concentrations.

c. Treatment is reserved for patients with toxic levels of HbM > 30%, which consists of intravenous infusion of methylene blue.

4. Glycosylated hemoglobin (HbA1c) is a minor component of adult Hb on chromatography analysis.
   a. Pathophysiology
      (1) A carbohydrate component is added to the N-terminus of the \( \beta \)-globin chain of Hb.
      (2) HbA1c is Hb with a glucose irreversibly attached.
         (a) Synthesis is proportional to the time-averaged concentration of blood glucose.
         (b) Older RBCs have a higher level of HbA1c than young RBCs.
         (c) Blood concentrations of glucose > 400 mg/dL significantly increase levels of HbA1c.
   b. Laboratory diagnosis
      (1) Methods include variations of chromatography.
      (2) HbA1c levels used as an indicator of control of blood glucose levels in diabetes.
      (3) Average levels are 7.5% in diabetes and 3.5% in nondiabetic patients.

IV. ERYTHROCYTES AND ERYTHROPOIESIS

A. Erythroid maturation
   1. Production begins with the multipotential stem cell (CFU-S). The differentiation of a stem cell, induced by certain microenvironmental influences, results in a committed erythroid progenitor cell.
   2. The committed unipotential cell compartment for erythropoiesis consists of two compartments, as defined by their behavior in cell culture systems.
      a. BFU-E stem cells (see Figure 3–14) maintain an active cell cycle maintained by a burst-promoting factor released by the local microenvironment.
         (1) BFU-E cells have a low concentration of erythropoietin (EPO) receptors and respond only to high concentrations of EPO.
         (2) T lymphocytes are required for optimal BFU-E growth.
      b. Differentiation of the BFU-E cell pool gives rise to the unipotential CFU-E stem cell pool (see Figure 3–14).
         (1) CFU-E cells have a high concentration of EPO membrane receptors; hence, they respond to low EPO concentrations.
         (2) EPO stimulation transforms the CFU-E cell into the earliest recognizable erythroid precursor, the pronormoblast.
   3. Pronormoblasts (rubriblasts) comprise the first recognizable erythroid precursor stage as well as the first hemoglobin-synthesizing cell. These blasts begin a controlled process of photoporphyrin production, globin-chain synthesis, iron uptake, and Hb assembly.
      a. Size. Pronormoblasts measure approximately 20 \( \mu m \) in diameter (the largest of erythroid precursors).
      b. Distinguishing morphologic and cytoplasmic characteristics are presented in Table 3–2.
      c. This is an actively mitotic stage, and its division forms two basophilic normoblasts.
   4. Basophilic normoblasts (prorubricytes) are slightly smaller in size than pronormoblasts.
      a. Distinguishing morphologic and cytoplasmic characteristics are given in Table 3–2.
      b. This is an actively mitotic stage, and its division gives rise to two polychromatic erythroblasts.
Table 3–2  Morphologic and Cytoplasmic Characteristics of Erythroid Cells

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Morphologic Characteristics</th>
<th>Cytoplasmic Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pronormoblasts</td>
<td>Fine, uniform chromatin pattern</td>
<td>Moderate in amount</td>
</tr>
<tr>
<td></td>
<td>Intensely staining chromatin</td>
<td>Moderately basophilic (bluish)</td>
</tr>
<tr>
<td></td>
<td>Prominent nuclear membrane</td>
<td>Granules are absent</td>
</tr>
<tr>
<td>Basophilic normoblasts</td>
<td>Coarse, partially clumped chromatin</td>
<td>Slightly basophilic due to a high amount of cytoplasmic RNA</td>
</tr>
<tr>
<td>(promoblasts)</td>
<td>with a wheel-spoke pattern</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Nuclear parachromatin stains pink</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Nucleoli are present, but not always visible</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cell borders often irregular because of pseudopodia</td>
<td></td>
</tr>
<tr>
<td>Polychromatic erythroblasts (rubricytes)</td>
<td>Nuclear volume occupies only half of the cell area.</td>
<td>Stains various shades of gray due to mix of RNA and hemoglobin</td>
</tr>
<tr>
<td></td>
<td>Intensely staining chromatin</td>
<td></td>
</tr>
<tr>
<td>Orthochromic normoblasts (metarubricytes)</td>
<td>Smaller normoblasts than previous stage. with nucleus.</td>
<td>Abundant hemoglobin</td>
</tr>
<tr>
<td></td>
<td>Last stage in maturation sequence</td>
<td>Slight polychromasia</td>
</tr>
<tr>
<td>Reticulocytes</td>
<td>Nuclear material extruded</td>
<td>Polychromatic due to remaining RNA</td>
</tr>
<tr>
<td></td>
<td>Immature reticulocytes are larger than RBCs</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Immature reticulocytes are sticky</td>
<td></td>
</tr>
</tbody>
</table>

Other characteristics include:

a. Macrophages phagocytose the extruded nuclear material.

b. The reticulocyte is released into the circulation after 2 days of maturation in the marrow.

c. Eight reticulocytes are normally produced from one pronormoblast.

d. Reticulocytes synthesize Hb for approximately 1 day after leaving the marrow.

e. Residual ribosomes, mitochondria, and other organelles are removed in the spleen or are internally dissolved. The result is a mature RBC, which has an average life span of 120 days.

8. Iron incorporation is a vital part of erythroid maturation.

a. Iron is transferred from transferrin into the young normoblasts, where it becomes attached to specific normoblast membrane receptors.

b. Free iron is shuttled into the normoblast, and the transferrin molecule is released for recirculation.
Intracellular iron is shuttled to the mitochondria for heme-ring production, or it is temporarily deposited in the cytoplasm as ferritin.

b. A secondary supply of iron is provided by a central macrophage through cell-to-cell delivery.


a. Megaloblastosis is an abnormal maturation of erythroid precursors resulting from a vitamin B₁₂ or folic acid deficiency.

b. Megaloblasts have an impaired ability to synthesize and replicate their own nuclear DNA. Intermitotic and mitotic phases of mitosis are prolonged and out of synchrony.

(1) As a result, nuclear maturation lags behind cytoplasmic maturation.

(2) The nuclear chromatin pattern of megaloblasts is more open, and breaking up of the nucleus is common (i.e., karyorrhexis).

(3) This results in an increased frequency of circulating RBCs with Howell-Jolly bodies, which are remnants of nuclear chromatin.

B. Regulation of erythrocyte production

1. The number of circulating RBCs may be regulated by changing the rate of production in the marrow or the rate of release from the marrow. This regulation is normally well balanced, because the rate of RBC destruction does not significantly vary.

2. Impaired oxygen transport to the tissues and low intracellular oxygen tension triggers RBC production in the marrow. Conditions that stimulate erythropoiesis include:

a. Anemia

b. Cardiac or pulmonary disorders

c. High altitudes

3. Oxygen tension in tissues is regulated, in part, by the oxygen affinity of Hb.

a. Modulation. Hemoglobin-oxygen affinity is modulated by the concentration of phosphates in the RBC.

(1) The primary phosphate is 2,3-DPG.

(2) 2,3-DPG combines with β chains of the reduced Hb molecule, which results in reduced Hb affinity for oxygen binding.

b. In areas of hypoxic tissue, as oxygen moves from Hb into the tissue, the amount of reduced Hb decreases. This results in:

(1) More 2,3-DPG being bound

(2) Lower Hb oxygen affinity, which results in more oxygen being released to the tissues

(3) If tissue hypoxia persists, the depletion of 2,3-DPG leads to increased glycolysis and production of more 2,3-DPG, which further lowers Hb oxygen affinity.

4. Reduced intracellular oxygen tension leads to the production of EPO.

a. Background. EPO is a glycoprotein hormone that controls RBC production at the level of the marrow.

(1) Production. EPO is produced mainly by kidney glomeruli, when cells in the glomerular tuft of the kidney experience a reduced oxygen tension.

(2) Location. EPO is present in the plasma and urine of all mammals, with a biologic half-life of 4 to 6 hours and a normal reference range of 22 to 54 milli-immunochemical units per milliliter (mU/mL).

b. Three main EPO marrow effects include:

(1) Stimulation of committed unipotential cells to proliferate and differentiate into pronormoblasts

(2) Shortening the generation time of each maturation stage

(3) Promoting early release of reticulocytes into the blood

c. The end result of EPO stimulation is an increased number of marrow normoblasts.

5. Other regulators of erythropoiesis

a. Plasma Hb may have a feedback stimulation of RBC production or result in increased EPO production.
b. Hemolytic anemias result in a higher reticulocyte count than other disorders that stimulate RBC production.

c. Androgens have a synergistic effect on EPO release and are thought to be the cause of higher red cell values in men.

C. Erythrocyte structure

1. General considerations. RBCs are biconcave disks with a mean diameter of 7 to 8 μm and a mean volume of 90 fL. The RBC has no nucleus or mitochondria and cannot synthesize protein but is packaged with enough limited metabolism to exist for 120 days.

a. RBCs have more surface area than volume, which creates a soft, pliable cell.

b. The life span of an RBC depends on the relationship between the RBC membrane and metabolism. An RBC must have good self-healing ability. Cell damage or injury can produce cell fragments rather than Hb leakage and cell lysis.

c. The main mission of an RBC is to carry oxygen and carbon dioxide to and from all tissues.

d. With cell aging, enzymatic failure leads to the loss of pliability and splenic destruction.

2. The RBC membrane is a lipid bilayer, two molecules thick, consisting of tightly packed phospholipid molecules.

a. Cholesterol is found in the bilayer in a 1:1 molar ratio with phospholipids.

(1) RBC membrane cholesterol is in rapid exchange with plasma unesterified cholesterol.

(2) Cholesterol content of the RBC membrane depends on the plasma concentration of cholesterol, free bile acids, and the esterifying enzyme lecithin cholesterol acyltransferase (LCAT).

(3) Changes in RBC shape and survival can result from changes in plasma lipids.

(a) Patients with hepatocellular disease or biliary obstruction have impaired LCAT activity.

(b) This results in cholesterol overloading of the RBC membrane and excess RBC membrane surface area.

c. Red cells appear as target cell forms and acanthocytes, which have a reduced survival compared with normal RBCs.

b. Membrane proteins are of two basic classes.

(1) Integral proteins provide anion channels through the RBC membrane.

(a) These proteins are in contact with both sides of the membrane.

(b) Oligosaccharide chains are attached to the external surface of transmembrane proteins, providing a negative charge to the RBC surface, which prevents autoagglutination of RBCs.

(2) Peripheral proteins provide a structural network on the inner surface of the membrane, giving the cell its biconcave structure. Peripheral proteins consist of spectrin, components 4.1 and 5, and actin. Abnormalities of these proteins are responsible for cell-shape deformities and hemolytic anemias (e.g., hereditary elliptocytosis, hereditary spherocytosis).

c. The outside layer of the RBC membrane contains numerous antigenic determinants, which provide a genetically determined “specific map” of the RBC surface.

(1) More than 300 RBC antigens have been identified, comprising approximately 15 genetically distinct blood group systems.

(2) RBC antigens are composed largely of the oligosaccharide groups of the integral proteins.

(3) Almost all antigenic groups are intrinsic parts of the membrane and appear during early cell development. The exception is the Lewis group, which is secondarily absorbed onto cell surfaces.

(4) Common RBC antigen systems include:

(a) ABO
(b) Secretor and Lewis
(c) H
Figure 3–15 The metabolism of the RBC is primarily from anaerobic glycolysis via the Embden-Meyerhof (EM) pathway and the pentose-phosphate shunt. ADP = adenosine diphosphate; ATP = adenosine triphosphate; DHAP = dihydroxyacetone phosphate; 1,3-DPG = 1,3-diphosphoglycerate; 2,3-DPG = 2,3-diphosphoglycerate; FDP = fructose-1,6-diphosphate; F6P = fructose-6-phosphate; G-P-isomerase = glucose-phosphate isomerase; G3P = glucose-3-phosphate; G6P = glucose-6-phosphate; GSH = glutathione (reduced form); GSSG = glutathione (oxidized); NAD = nicotinamide-adenine dinucleotide; NADH = nicotinamide-adenine dinucleotide (reduced form); NADP = nicotinamide-adenine dinucleotide phosphate (reduced form); pentose-5-P = pentose-5-phosphate; PEP = phosphoenolpyruvate; 2-PG = 2-phosphoglycerate; 3-PG = 3-phosphoglycerate; 6-PG = 6-phosphogluconate. (Reprinted from Besa E, Catalano PM, Kant J, et al. Hematology. Baltimore: Williams & Wilkins; 1992:98 with permission.)

(d) MN and P
(e) Rh, Kell, and Duffy

D. Erythrocyte metabolism
1. The RBC has no nucleus or mitochondria to metabolize fatty and amino acids for the provision of energy substrates.
2. Energy metabolism in the RBC is almost exclusively through the breakdown of glucose.
3. Three basic metabolic pathways are found in the RBC (Figure 3–15).
   a. Embden-Meyerhof (EM) pathway is a nonoxidative anaerobic pathway that handles 90% of glucose utilization in the RBC.
      (1) The end result of the pathway is the net production of 2 adenosine triphosphate (ATP).
      (a) ATP is necessary for the survival of an RBC because ATP:
          (i) Maintains cell shape and flexibility
(ii) Energizes the metabolic pumps that control cellular sodium, potassium, and calcium flux
(iii) Preserves membrane lipids
(b) Deficiencies in ATP, due to an inherited or acquired defect in glycolysis, can reduce cell survival and result in hemolytic anemia.

(2) The EM pathway also plays an essential role in maintaining pyridine nucleotides in a reduced state (i.e., nicotinamide adenine dinucleotide, reduced form (NADH)) to support the conversion of methemoglobin (HbM) to hemoglobin in the methemoglobin reductase pathway.

(a) HbM results from the oxidation of iron (Fe) from ferrous ion (Fe$^{2+}$) to ferric ion (Fe$^{3+}$).
(b) HbM has a very low oxygen binding affinity.
(c) The accumulation of HbM greatly reduces the oxygen-carrying capacity of the RBC.
(d) The methemoglobin reductase pathway counteracts the oxidized state by reducing Fe$^{3+}$ to Fe$^{2+}$.
(e) This pathway relies on the reducing capacity of nicotinamide adenine dinucleotide (NAD).
(f) Persons homozygous for an abnormal methemoglobin reductase gene accumulate 20% to 40% Hb M in RBCs.
(g) Persons with heterozygous enzyme deficiency maintain sufficient HbM under normal conditions but are susceptible to hemolysis by oxidizing drugs.

b. The Luebering-Rapaport pathway is necessary for the production of 2,3-DPG.
(1) The amount of 2,3-DPG produced at any one time depends on the glycolysis rate-limiting enzyme, phosphofructokinase.
(2) 2,3-DPG production also depends on an adequate supply of inorganic phosphate.
(3) 2,3-DPG binds to Hb and decreases the oxygen affinity of Hb, thereby releasing more oxygen to the tissues.

E. Erythrocyte life cycle
1. A constant red cell mass in the circulation is ensured by the balance between delivery of RBCs from the marrow to the blood and the removal of aged or abnormal RBCs from the circulation.
2. As RBCs age, catabolic changes occur that result from cellular enzyme depletion, which leads to a decrease in cell flexibility.
   a. Cell rigidity makes it more difficult for RBCs to get through small capillaries or splenic sinusoids. The 2 to 3 μm-fenestrations of the splenic sinusoids remove aged or abnormal RBCs that have a higher degree of rigidity.
   b. Splenic trapping results in cell lysis and monocyte/macrophage phagocytosis of debris.
3. Extravascular destruction is removal of RBCs by the spleen and liver (i.e., reticuloendothelial system). This pathway is the most efficient method of cell removal and recovery of essential components such as amino acids and iron.
   a. Intramacrophage RBC breakdown occurs following phagocytosis, when the RBC is attacked by lysosomal enzymes. Hb is broken down by an enzyme known as heme-oxygenase.
b. Iron is released from the heme group, returned to plasma transferrin, and transported back to the erythroid marrow. Small amounts of iron can be stored within the reticuloendothelial macrophage as ferritin or hemosiderin.

c. Amino acids from globin chains are redirected to the body’s amino acid pool.

d. The photoporphyrin pyrrole ring is broken down at the $\alpha$-methane bridge, and its $\alpha$-carbon is exhaled as CO. 

   (1) The opened tetrapyrole, bilirubin, is carried by plasma albumin in the unconjugated (i.e., the indirect bilirubin) form to the liver.

   (2) Bilirubin is conjugated in the liver to form bilirubin glucuronide (i.e., direct bilirubin).

e. Conjugated bilirubin is excreted from the liver into the small intestine via the bile duct, where it is converted by bacterial flora to urobilinogen.

   (1) Most urobilinogen is excreted in the stool as urobilin.

   (2) Between 10% and 20% of urobilinogen is reabsorbed by the gut. The reabsorbed urobilinogen is either excreted in urine or returned to the gut via an enterohepatic cycle.

   (3) With liver disease, the enterohepatic cycle is impaired, and an increased amount of urobilinogen is excreted in the urine.

4. Intravascular destruction of RBCs accounts for <10% of RBC loss, but it can increase in certain hemolytic diseases. Free hemoglobin is disposed of in the following manner:

a. The free hemoglobin tetramer is unstable in plasma; therefore, tetramers are quickly dissociated into $\alpha_1\beta_1$ dimers.

b. Hemoglobin dimers are quickly bound to plasma haptoglobin.

   (1) Haptoglobin binding stabilizes the home-globin bond and prevents renal excretion of Hb.

   (2) The haptoglobin-hemoglobin complex is removed from the circulation by reticuloendothelial macrophages and is processed intracellularly in the same manner as extracellular RBC destruction.

   (3) There is a limited supply of plasma haptoglobin, so the number of haptoglobin-hemoglobin complexes that can be formed acutely is limited.

   (a) A sudden release of several grams of Hb intravascularly can exceed the haptoglobin-binding capacity.

   (b) When the haptoglobin-hemoglobin complex is processed in the macrophage, the haptoglobin itself is also catabolized.

   (c) A decrease or absence of serum haptoglobin may be used to indicate increased intravascular hemolysis.

c. If haptoglobin is depleted, unbound Hb dimers are free to be filtered by the renal glomerulus.

   (1) As much as 5 g/day can be reabsorbed by renal tubular epithelial cells and converted to hemosiderin for storage.

   (2) If the amount of free Hb is high, the tubular uptake capacity can be exceeded. Then, dimers are excreted in the urine as free Hb.

   (3) Large amounts of filtered Hb can be destructive to renal tubular cells.

   (4) A large amount of Hb excretion is accompanied by the excretion of hemosiderin and iron loss.

d. Part of the free Hb not bound to haptoglobin may be oxidized to methemoglobin.

   (1) The heme rings dissociate from the globin chains and are bound to another transport protein, hemopexin.

   (2) Heme-hemopexin complexes are cleared from the circulation by reticuloendothelial macrophages and catabolized.

   (3) A small percent of the heme groups are bound to albumin as methemalbumin.

F. Measurements of RBC production and destruction

1. Anemia occurs when delivery of RBCs to the circulation is decreased or when the removal of RBCs from the blood is increased and cannot be compensated for by increased marrow production (see Chapter 4).
Anemia can result from a marrow production defect or from an RBC survival disorder (e.g., hemolysis). Therefore, the correct laboratory tests can determine the source and cause of most anemias. TIBC = total iron-binding capacity; LDH = lactate dehydrogenase.

a. When anemia develops, the end result is tissue hypoxia.
   (1) Hypoxia stimulates increased erythropoietin production by the kidneys.
   (2) Increased EPO levels result in a normoblastic hyperplasia, which produces more RBCs to be released into the circulation.

b. In a normal person, the marrow is capable of 6 to 8 times the normal output.

2. Measurement of the total erythron must take into account the balance between marrow production, survival in the circulation, and destruction in the hepatic and splenic macrophage (Figure 3–16).

3. Measurement of total RBC production can be obtained from an absolute measure of the number of immature erythroid cells through the injection and tracking of radioactive iron.

4. Measurements of Hb catabolism and RBC survival can be obtained with several methods.
   a. An indication of the amount of heme breakdown can be determined by the amount of exhaled CO.
   b. Urobilinogen can be measured in the stool.
   c. Serum bilirubin and lactate dehydrogenase (LDH) levels can provide a quantitative indication of RBC turnover. Hemolytic disorders can result in high levels of bilirubin and LDH.
   d. RBC survival can be estimated by removing a sample of whole blood and labeling it with the radioisotope chromium 51 ($^{51}$Cr-labeled).

5. The functional capacity of the erythroid marrow can be measured with several methods.
   a. Relative numbers of marrow stem cells may be estimated using cell culture techniques.
   b. The density of the erythroid marrow can be estimated from a bone marrow examination and by obtaining a M:E ratio (see Section II D 3 d (3)(b)).
      (1) The normal adult M:E ratio ranges from 2:1 to 4:1.
      (2) A hemolytic disorder may display an M:E of 1:1, which indicates an erythroid hyperplastic marrow.
      (3) An erythroid hypoplastic marrow may demonstrate a M:E ratio >4:1.
   c. The reticulocyte count is a good way to effectively measure the production rate of RBCs.
      (1) Background. Because RBC intracellular RNA disappears after about a day in the circulation, the reticulocyte count provides a rough measurement of the number of RBCs being delivered by the marrow to the circulation each day.
      (2) Method. Basically, a reticulocyte count is obtained by staining RBCs with a vital stain known as new methylene blue.
         (a) Blood smears of stained RBCs are made, and a set number of RBCs are counted while the total number of reticulocytes is simultaneously counted.
         (b) Reticulocytes are reported as a percent of RBCs.
         (c) Normal values range from 0.5% to 2.0%.
<table>
<thead>
<tr>
<th>Hematocrit (%)</th>
<th>Reticulocyte Maturation Time (D)</th>
</tr>
</thead>
<tbody>
<tr>
<td>45</td>
<td>1.0</td>
</tr>
<tr>
<td>35</td>
<td>1.5</td>
</tr>
<tr>
<td>25</td>
<td>2.0</td>
</tr>
<tr>
<td>15</td>
<td>2.5</td>
</tr>
</tbody>
</table>

(3) If the total RBC count is also determined, the absolute reticulocyte count can be determined by multiplying the percent reticulocytes by the RBC count.

(a) The normal absolute reticulocyte count ranges from 25,000 to 75,000/mm³.

(b) The formula for obtaining the absolute reticulocyte count is:

\[
\text{Absolute Reticulocyte Count} = \left( \frac{\text{Percent Reticulocytes}}{100} \right) \times \left( \frac{\text{RBCs} \times 10^6}{\text{mm}^3} \right)
\]

(4) Because the normal maturation time for reticulocytes in the circulation is 1 day, the normal RBC production is an average of 50,000/mm³/d.

(5) Often, an increased circulatory maturation time of reticulocytes, which is caused by accelerated release from the marrow, must be taken into account.

(a) Even with increased erythropoiesis, reticulocytes still need 2 to 3 days to mature.

(b) To avoid an overestimation of daily RBC production, a correction factor is used based on the estimated maturation time of reticulocytes in the circulation.

(i) The finding of polychromatic RBCs or nucleated RBCs (NRBCs) on the blood smear indicates accelerated erythropoiesis, a shift reticulocytosis, and a need to correct the reticulocyte count for circulating maturation time with the maturation index.

(ii) The maturation index varies inversely with HCT (Table 3–3).

(c) Example. A patient is seen with a HCT of 25%, an RBC count of 2.89 \times 10^6/mm³, and a reticulocyte count of 7%. The calculated absolute reticulocyte count of this patient is:

\[
0.07 \times (2.89 \times 10^6) = 202 \times 10^3/\text{mm}^3
\]

(i) Because the average normal reticulocyte count is 50,000, this patient has the following rate of reticulocyte production:

\[
\frac{202,000}{50,000} = \text{four times normal amount}
\]

(iii) When corrected for maturation time using the patient’s HCT of 25% (Table 3–3), there are 4 ÷ 2 = two times as many reticulocytes per day as normal.

(d) An alternate method used to correct for shift reticulocytosis is by calculating the reticulocyte index (RI).

(i) Example. Using the same patient values as before, the formula for obtaining an RI is:

\[
\text{RI} = \frac{\text{Percent Reticulocytes}}{\text{Normal HCT}} \times \text{Patient’s HCT (25%)}
\]

(ii) After the RI is known, the reticulocyte production index (RPI) can be determined:

\[
\text{RPI} = \frac{\text{RI} \times \text{Maturation time (2 days)}}{2 \times \text{as many reticulocytes/day}}
\]

6. Measurement of blood volume might be necessary for the diagnosis of certain disorders such as anemias or polycythemia (i.e., increase in RBC volume).
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a. Substances that combine with RBCs (e.g., chromium 51) can be used to directly measure the total red cell volume. Then, the plasma and whole blood volumes can be calculated from the HCT value.

b. Substances that combine with plasma proteins can be used to directly measure plasma volume. Then, the red cell and whole blood volumes can be calculated from the HCT value.

(1) Evans’s blue dye binds to plasma proteins.

(2) Radio-iodine saturated albumin (RISA) mixes with native albumin to provide a measure of plasma volume.

G. RBC morphology: normal and abnormal

1. Size variations in RBCs can have a diagnostic implication and are important to report.

a. Anisocytosis is a term indicating RBC variation in size (Web Color Image 3–1).

(1) Anisocytosis is usually quantitated as 1+, 2+, 3+, or 4+. There must be a variation in size of more than 10% of erythrocytes per high-power field for a grading of 1+.

(2) When possible, use the RDW to aid in recognition and quantitation of anisocytosis. An RDW > 15.0 indicates anisocytosis.

b. Normocytosis of RBCs indicates relatively uniform size (i.e., < 10% variation).

c. Macrocytosis is a term used to indicate the presence of large RBCs with a mean diameter > 8 μm. Refer to Web Color Image 3–2.

(1) Macrocytosis should be quantitated in the following manner:

(a) 1+ (i.e., slight) if there are approximately 25% macrocytic RBCs present per high-power field

(b) 2+–3+ (i.e., moderate) if there are 25% to 50% macrocytic RBCs present per high-power field

(c) 4+ (i.e., marked) if there is > 50% macrocytic RBCs per high-power field

(2) When possible, use the patient’s MCV to aid in a quantitation of macrocytosis. An MCV > 100 fl indicates macrocytosis.

(3) Macrocytosis has the following diagnostic implications:

(a) Megaloblastic anemias

(b) Shift reticulocytosis (e.g., hemolysis)

(c) Anemia of liver disease

d. Microcytosis should be quantitated in a manner similar to the quantitation of macrocytosis. If the MCV is not available, finding a small lymphocyte provides a comparable scale. Refer to Web Color Image 3–3.

(1) An MCV < 80 fl indicates microcytosis.

(2) Microcytosis has the following diagnostic implications:

(a) Iron-related disorders

(b) Sideroblastic anemias

(c) Anemia of chronic disorders

(d) Thalassemias

2. The color of the RBCs can be related to the Hb content of the cells.

a. Normochromic RBCs have a normal red color and Hb content. If possible, the patient’s MCH and MCHC should be used to provide guidelines for RBC color appearance.

b. Hypochromia refers to RBCs that show a less than normal amount of hemoglobin staining, and the cells’ central pallor is increased to more than one third of the cell diameter (Web Color Image 3–4). The patient’s MCH and MCHC should be used to provide guidelines for RBC color appearance. Hypochromia should be graded in the following manner:

(1) 1+ (i.e., slight) if RBCs show a central pallor occupying one third to two thirds of the cell’s diameter

(2) 2+–3+ (i.e., moderate) if RBCs show a central pallor occupying more than two thirds of the cell’s diameter

(3) 4+ (i.e., marked) if RBCs show red hemoglobin staining that appears only as a rim on the periphery of the cell
3. Shape changes often have a diagnostic implication and should be graded as slight, moderate, or marked (i.e., 1+, 2+, or 3+) per microscopic field at a magnification of 1000X.

a. Poikilocytosis refers to RBC variation in shape. There must be a variation in shape of more than 10% of the RBCs per high power field for a grading of 1+. Refer to Web Color Image 3–5.

b. Spherocytosis indicates RBCs of spherical shape (Web Color Image 3–6). (1) Spherocytes appear microcytic and hyperchromic, because of their spherical form. The central pallor is absent (i.e., the typical doughnut shape is absent). (2) Grading. Spherocytosis should be graded as slight, moderate, or marked (i.e., 1+, 2+, 3+) using the same guidelines previously described for changes in size. (3) Spherocytes will be normocytic with a normal MCV. (4) Diagnostic implications of spherocytes are as follows: (a) Hereditary spherocytosis (b) Hemolysis
c. Ovalocytosis (i.e., elliptocytosis) indicates red cells that vary in shape from slightly oval to pencil-shaped or cigar-shaped (Web Color Image 3–7). (1) Ovalocytosis is reported if more than 10% of RBCs per microscopic field are oval or elliptical in shape. (2) The diagnostic implications of ovalocytosis are as follows: (a) Hereditary elliptocytosis (b) Megaloblastic anemias
d. Sickle cells (i.e., drepanocytes) are flattened and elongated cells that are often curved and may have the appearance of a curved blade (i.e., sickle) with sharp points (Web Color Image 3–8). The presence of sickle cells in any amount suggests an investigation of sickle cell anemia.
e. Burr cells are irregularly shaped RBCs with symmetric, knobby projections. Refer to Web Color Image 3–9. (1) Burr cells are produced by a rapture of the cell membrane by enlarged cytoplasmic vacuoles. (2) Care must be taken to distinguish burr cells from crenated RBCs, which are produced by too rapid a drying of the blood smear. (3) Diagnostically, burr cells implicate renal disease.
f. Schistocytes (i.e., fragmented RBCs) are pieces of RBCs that are small and triangular with pointed ends. Schistocytes are commonly found in patients with hemolytic anemia and severe burns. Refer to Web Color Image 3–10.
g. Tear drop cells (i.e., dacryocytes) have one pointed end and a round body. Refer to Web Color Image 3–11. Tear drop RBCs have the following diagnostic implications: (1) Megaloblastic anemias (2) Myelofibrosis with myeloid metaplasia (3) Acquired hemolytic anemias
h. Acanthocytes are irregularly shaped RBCs with asymmetric, sharp projections (Web Color Image 3–12). (1) Acanthocytes are produced by cholesterol overloading of the membrane. (2) Diagnostically, acanthocytes implicate chronic liver disease.

4. Abnormal forms of RBCs should be graded as slight, moderate, or marked (i.e., 1+, 2+, or 3+) per microscopic field at a magnification of 1000X.

a. Target cells (i.e., leptoocytes) are flattened RBCs that reveal peripheral and central zones of Hb, giving the appearance of a target (Web Color Image 3–13). (1) Target cells are produced by cholesterol overloading of the membrane. (2) The diagnostic implications of target cells are as follows: (a) Chronic liver disease (b) Thalassemias (c) Hemoglobinopathies
b. **Polychromatophilia** (i.e., polychromasia) refers to an increase in the number of younger RBCs (e.g., reticulocytes) with incomplete hemoglobinization. Refer to Web Color Image 3–14.

(1) These cells are larger than normal RBCs, lack a central pallor, and stain a pale blue.

(2) Polychromasia should be quantitated in the following manner:
- **1+** if 1–3 polychromatic cells are found per microscopic field
- **2+** if 3–5 polychromatic cells are found per microscopic field
- **3+** if more than five polychromatic cells are found per microscopic field

c. **Basophilic stippling** describes cytoplasmic inclusions. RBCs have cytoplasm that is stippled with a number of fine blue granules, which are ribosomal-RNA remnants (Web Color Image 3–15).

(1) Stippled cells are quantitated in the following manner:
- **Slight** if one stippled RBC is noted in every other microscopic field
- **Moderate** if 1–2 stippled RBCs are noted in every microscopic field
- **Marked** if three or more stippled RBCs are noted in every microscopic field

(2) The diagnostic implications of basophilic stippling are as follows:
- **Lead poisoning**
- **Toxin poisoning**
- **Conditions that greatly accelerate erythropoiesis**

d. **Cabot rings** are thready, blue, ring-shaped, twisted, or figure-eight RBC cytoplasmic inclusions. They are seen rarely in severe anemia.

e. **Howell-Jolly Bodies** are dark violet-staining DNA remnants in RBCs (Web Color Image 3–16).

(1) These inclusions usually occur singly and average 1 μm in diameter.

(2) The diagnostic implications of Howell-Jolly bodies are as follows:
- **Splenectomy**
- **Hemoglobinopathies**
- **Severe hemolysis**

f. **NRBCs** are usually orthochromic normoblasts, but can represent any stage of erythroid maturation (Web Color Image 3–17).

(1) Their presence in the peripheral circulation is indicative of marrow stimulation.

(2) NRBCs are normally found in newborns.

(3) They are reported as the number of NRBCs counted per 100 WBCs.

(4) The diagnostic implications of NRBCs are as follows:
- **Acute hemorrhage**
- **Congestive heart failure**
- **Hypoxia**
- **Hemolytic anemia**
- **Leukemias**
- **Megaloblastic anemias**
- **Myelofibrosis**

(5) **Pappenheimer bodies** are RBC cytoplasmic inclusions measuring up to 2 μm in diameter (Web Color Image 3–18). One to 10 may be seen in a single RBC. The granules are **free iron** and stain dark violet with Wright’s stain.

(1) Particles also stain positive with an iron stain (i.e., **Prussian blue**).

(2) The presence of Pappenheimer bodies indicates abnormal hemoglobin synthesis.

(3) The diagnostic implications of Pappenheimer bodies are as follows:
- **Severe hemolytic anemia**
- **Asplenia and post-splenectomy state**
- **Sideroblastic anemia**
- **Thalassemias**
- **Megaloblastic anemia**
Rouleaux formation is reported if the RBCs become aligned in aggregates resembling stacks of coins. Rouleaux are often seen as an artifact in thick areas of the blood film. Refer to Web Color Image 3–19.

1. Rouleaux are quantitated in the following manner:
   a. Slight, if one to two RBC chains are found per thin microscopic field
   b. Moderate, if three to four RBC chains are found per thin microscopic field
   c. Marked, if five or more RBC chains are found per thin microscopic field

2. The diagnostic implication of rouleaux is often associated with hyperproteinemia disorders such as multiple myeloma.
CHAPTER 4
Hematologic Disorders

JOEL HUBBARD, PhD, MT (ASCP)

I. RED BLOOD CELL INDICES AND THEIR USE IN THE DIAGNOSIS OF ANEMIA

A. Red blood cell (RBC) indices, known as corpuscular constants, are provided by the majority of automated cell counters. Indices can also be calculated individually from the RBC count, hemoglobin (Hb), and hematocrit (Hct) values.

B. These parameters are useful in the diagnosis, classification, and differentiation of anemias, as seen in Table 4–1. They can also provide useful guidelines in assessing blood smear RBC morphology.

C. Individual corpuscular constants include the mean cell volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC).

1. MCV is defined as the mean or average size (in cubic microns) of the individual erythrocyte.
   a. The MCV may be calculated from the volume and number of erythrocytes in a given quantity of blood (Box 4–1). The formula for calculation is as follows:

   \[
   \text{MCV (fL)} = \frac{\text{Hct} \times 10}{\frac{\text{RBCs} \times 10^6}{\text{mm}^3}}
   \]

   b. The normal MCV for an adult is 80 to 100 fl.

   (1) If the cells are larger than normal, the MCV is increased, and the condition is called macrocytosis. If the condition is associated with anemia, it is called a macrocytic anemia.

   (2) If the cells are smaller than normal, the MCV is decreased, and the condition is called microcytosis (or microcytic anemia if associated with anemia).

2. MCH is the mean or average amount of Hb by weight per cell, expressed in micromicrograms (μμg) or picograms (pg).
   a. Calculation. The MCH may be calculated from the Hb and the Hct values (Box 4–2). The formula for calculation is as follows:

   \[
   \text{MCH (pg)} = \frac{\text{Hb g/dL} \times 10}{\frac{\text{RBCs} \times 10^6}{\text{mm}^3}}
   \]

   b. The normal MCH for the adult is 29 ± 2 pg.

   (1) In macrocytic erythrocytes, the amount of Hb may be greater than normal. The increase in Hb parallels the increase in cell size.

   (2) The RBC is never supersaturated with Hb. There is no such thing as a hyperchromic RBC.
A Concise Review of Clinical Laboratory Science

Table 4–1 Morphologic Classification of Anemia and RBC Morphology by Indices Range

<table>
<thead>
<tr>
<th>RBC Morphology</th>
<th>MCV (fL)</th>
<th>MCH (pg)</th>
<th>MCHC (%)</th>
<th>Anemias</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normocytic/normochromic</td>
<td>80–100</td>
<td>27–31</td>
<td>32–36</td>
<td>Acute blood loss</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Hemolytic anemia</td>
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<tr>
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<td></td>
<td></td>
<td></td>
<td>Aplastic anemia (early stage)</td>
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<td></td>
<td></td>
<td></td>
<td>Myelophthisic anemia</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Stem cell-related anemias</td>
</tr>
<tr>
<td>Macrocytic/normochromic</td>
<td>High</td>
<td>High</td>
<td>Normal</td>
<td>Megaloblastic anemia</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Anemia of liver disease</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>Chronic aplastic anemia</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>Acute hemolytic anemia (with shift</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>reticulocytosis)</td>
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<tr>
<td>Microcytic/normochromic</td>
<td>Low</td>
<td>Normal</td>
<td>Normal</td>
<td>Anemia of chronic inflammation</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>Iron deficiency anemia</td>
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<td></td>
<td>Thalassemia</td>
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<tr>
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<td>Lead poisoning</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>Porphyrias</td>
</tr>
<tr>
<td>Microcytic/hypochromic</td>
<td>Low</td>
<td>Low</td>
<td>Low</td>
<td>Sideroblastic anemia</td>
</tr>
</tbody>
</table>

*MCH = mean corpuscular hemoglobin; MCHC = mean corpuscular hemoglobin concentration; MCV = mean cell volume; RBC = red blood cell.*

Indices shown as normal range.

3. MCHC is the mean or average Hb concentration (in grams) per 100 mL of packed erythrocytes.

a. Calculation. The MCHC is calculated by dividing the Hb in grams per 100 mL (g/dL) of blood by the volume of packed erythrocytes per 100 mL of blood and multiplying by 100 (Box 4–3). The formula for calculation is as follows:

\[
\text{Hb g/dL} \times \frac{Hct \%}{100} = \text{MCHC g/dL}
\]

b. Normal range for adults is 34 +/- 2%. RBCs in which the MCHC is found decreased are termed hypochromic. The only pathologic condition in which the MCHC may be increased is spherocytic anemia. The MCHC also increases in the presence of cold agglutinins and agglutinated RBCs.

II. RED BLOOD CELL (RBC) DISORDERS can be classified as either polycthyemias or anemias. Polycythemias are disorders that have an increase in circulating RBCs, and therefore, an increased Hct. Anemias are disorders that have a decrease in circulating RBCs; therefore, these disorders have a decreased Hct.

A. Polycythemias are characterized by an increase in Hct >53% in men and 51% in women.

1. Clinical symptoms are caused by hypervolemia or hyperviscosity.

a. Hyperviscosity can cause sluggish flow of blood and a tendency toward thrombosis and disseminated intravascular coagulation (DIC).

Box 4–1 Calculation of Mean Cell Volume (MCV)

**EXAMPLE:**

Calculating the MCV of a patient with an RBC count of 5.0 × 10⁶/mm³ and a Hct of 45%:

\[
\text{MCV} = \frac{45 \times 10}{5.0} = 90 \text{ fL}
\]
Box 4–2 Calculation of Mean Corpuscular Hemoglobin (MCH)

EXAMPLE:
Calculating the MCH for a patient who has a Hb of 15 g/dL and an RBC count of 5.0 \( \times \) 10\(^6\)/mm\(^3\):

\[
\text{MCH} = \frac{15 \times 10^5}{5.0} = 30 \text{ fL}
\]

1. The decreased oxygen flow to tissues is compensated for by the higher Hct and increased blood volume (i.e., hypervolemia), which increases vessel diameter and therefore results in increased tissue perfusion.
2. This is not a benefit, however, because cardiac work is increased to deliver the same amount of oxygen as normal.

b. Hypervolemia increases blood-vessel diameter and therefore results in increased tissue perfusion. This can cause increased cardiac work, which is dangerous for an individual who is predisposed to heart problems.

2. Types of polycythemia include relative and absolute. Absolute polycythemia must be differentiated from relative polycythemia before treatment can ensue.
   a. Relative polycythemia refers to a condition in which the total RBC mass is normal but the Hct is elevated because the plasma volume is decreased.
      (1) Relative polycythemia can be caused by acute dehydration resulting from severe diarrhea, burns, or chronic diuretic therapy.
      (2) Spurious (false) polycythemia is related to chronic smoking. Nicotine induces a loss of plasma volume.
   b. Absolute polycythemia refers to an increase in the total RBC mass in the body. This is a true erythrocytosis caused by marrow erythroid hyperplasia or, secondarily, by an increase in erythropoietin (EPO), as illustrated in Figure 4–1.

B. Anemias are characterized by a reduced Hb concentration of <12 g/dL in men and 11 g/dL in women.
1. Clinical symptoms are caused by a decreased oxygen-carrying capacity of the blood and tissue hypoxia.
2. The physiologic effects of anemia include the following:
   a. A decreased Hb oxygen-affinity state results in a right shift of the oxygen dissociation curve.
   b. Decreased tissue perfusion in select areas of the body is caused by redistribution of blood flow favoring the more oxygen-dependent tissues, such as the brain and myocardium.
   c. Increased cardiac output results in anemic patients to maintain adequate tissue oxygen tension.
   d. Increased RBC production by the marrow results from an increase in EPO release from the kidneys because of a decrease in oxygen tension.
      (1) A compensatory increase in marrow erythropoietic cellularity occurs 4–5 days following a decrease in Hb values.
      (2) Erythroid hyperplasia in the marrow results in increased reticulocyte count and nucleated red blood cells (NRBCs) in the circulation.

Box 4–3 Calculation of Mean Corpuscular Hemoglobin Concentration

EXAMPLE:
Calculating the MCHC of a patient who has a Hb of 15 g/dL and a Hct of 45%:

\[
\text{MCHC} = \frac{15 \times 100}{45} = 33 \text{ g/dL}
\]
3. Types of anemias
   a. Absolute anemias result from impaired RBC production, blood loss, or accelerated RBC destruction (hemolysis).
      (1) Physiologic causes include:
          (a) Stem cell disorders
          (b) DNA disorders
          (c) Heme and globin disorders
          (d) RBC survival disorders (i.e., hemolytic disorders)
      (2) Morphologic classifications (Table 4–1) include the following (see Chapter 3):
          (a) Macrocytic
          (b) Microcytic
          (c) Normocytic
   b. Relative anemia can result from an increase in plasma volume rather than a decrease in the number of RBCs.
      (1) A dilutional anemia can occur by the third trimester of pregnancy.
      (2) Macroglobulinemia and multiple myeloma are associated with an increase in plasma globulin and protein concentration. This increase in plasma protein causes hyperosmotic plasma, and a dilutional anemia is caused by a compensatory increase in plasma volume.

III. ANEMIAS AND POLYCYTHEMIAS are RBC disorders that result from an imbalance in marrow multipotential or unipotential stem cell production and maturation. The imbalance is seen as either an increase in cellular production (i.e., polycythemia) or a decrease in cellular production, which results in an anemia.
CHAPTER 4 Hematologic Disorders

A. Polycythemias (Figure 4–1)

1. Primary absolute polycythemia is characterized by pancytosis or pancytopenia (i.e., all cell lines either increased or decreased). Initially, proliferation of erythroid, myeloid, and megakaryocytic bone marrow elements is uncontrolled. Polycythemia vera is a clonal disorder that results in failure of the multipotential stem cell.

   a. Laboratory characteristics of polycythemia vera include:
      (1) Increased RBC count and Hct
      (2) Increased bone marrow erythroid iron stores, which trap and deplete tissue iron stores
      (3) Decreased to absent EPO levels
      (4) Increased white blood cell (WBC) count, which is reflected by an increase in levels of serum and urine muramidase and serum vitamin B₁₂ and vitamin B₁₂ binding proteins
      (5) Thrombocytosis
   b. Patients who have polycythemia vera are commonly observed to have the clinical characteristics of splenomegaly and spontaneous hemorrhaging due to thrombosis and hyperviscosity.
   c. Treatments for polycythemia vera include the following:
      (1) Therapeutic phlebotomy is used to reduce the growing RBC volume. Therapeutic phlebotomy, however, further depletes tissue iron stores and can result in an iron-deficient (i.e., microcytic/hypochromic) erythrocytosis.
      (2) Splenectomy is used to relieve symptoms caused by splenomegaly and blood pooling.
      (3) Chemotherapy is used to treat patients who have advanced disease to kill rapidly growing malignant stem cells.
   d. Polycythemia vera can evolve into myelofibrosis or a myeloid metaplasia following chemotherapeutic treatment. Between 10% and 15% of patients progress to acute myelogenous leukemia.

2. Secondary absolute polycythemia has a normal WBC and platelet count and can be classified as:
   a. Polycythemia caused by appropriate EPO production
   b. Polycythemia caused by inappropriate EPO production
   c. Familial polycythemia, which is seen in children who have functionally normal Hb, Hb-oxygen saturation, and cardiopulmonary dysfunction
      (1) These children have high EPO levels unrelated to Hb concentration.
      (2) The uncontrolled EPO production is most likely caused by a defect in the regulation of EPO production.
      (3) Another variation of this genetic disorder is an inherited deficiency of 2,3-diphosphoglycerate phosphatase activities, which results in polycythemia.

B. Anemias (Figure 4–2) caused by a defect in stem cell erythropoiesis most commonly result from a depression of colony-forming units-erythroid (CFU-E) and burst-forming units-erythroid (BFU-E) unipotential stem cell maturation and differentiation.

1. Anemia of chronic disorders is usually a mild form of anemia occurring secondary to a chronic inflammatory disorder, such as an infection, rheumatoid arthritis (RA), or neoplastic diseases.
   a. RBC production by the marrow is normal but insufficient to compensate for a decreased RBC survival.
   b. The marrow CFU-E is capable of responding to EPO, but EPO production is low.
   c. This anemia is partially caused by a defect in iron metabolism that results from a block in the secondary iron storage system.
      (1) Monocytes and macrophages have a reduced ability to move stored intracellular iron to the erythroid marrow cells.
      (2) This recycling block results in a low serum iron level and an increase in the storage iron in tissue macrophages.
      (3) Systemic inflammation results in the release of interleukin-1 (IL-1) from macrophages, which blocks macrophage iron release. This decreases the reutilization of recycled iron that is released from heme catabolism.
Figure 4-2 Algorithm for the differential diagnosis of iron deficiency anemia from other microcytic/hypochromic anemias. MCHC = mean corpuscular hemoglobin concentration; MCV = mean cell volume; RBC = red blood cell; RDW = red cell distribution width; TIBC = total iron-binding capacity.

**d. Laboratory characteristics** of anemia of chronic disorders include the following:

1. Typically normocytic/normochromic RBCs that can be microcytic/hypochromic in some cases
2. Slight anisocytosis and poikilocytosis
3. Normal reticulocyte count
4. Unaltered WBC and platelet counts, except alterations resulting from the causative disease
5. Marrow is normocellular, but sideroblasts are decreased.
6. Serum iron is decreased, and total iron binding capacity (TIBC) is normal or decreased.

**2. Anemia of renal insufficiency** occurs in patients who have end-stage renal disease.

a. There is a general correlation between the severity of the anemia and the degree of elevation of the blood urea nitrogen (BUN). When BUN is >100 mg/dL, the Hct is usually <30%.

b. The primary cause of the anemia is a decreased production of EPO by the damaged kidney.

1. Ineffective erythropoiesis also results because of an impaired ability of the CFU-E to respond to EPO.
2. RBC survival is also decreased because of hemolysis that results from the damaging effects of uremic plasma.
   a. Hemolysis caused by uremia results in the formation of **burr cells**.
   b. **Burr cells and RBC fragments** are seen in the blood of a patient who has hemolytic-uremic syndrome.
   c. Increased blood urea levels also affect the functional ability of **platelets**.

Bleeding is a common problem that further amplifies the anemia.
3. Anemia of liver disease is characterized by shortened RBC survival and inadequate RBC production that occurs secondary to a chronic liver disease.
   a. RBC morphology is usually macrocytic or normocytic with target cells or acanthocytes caused by increased surface membrane cholesterol.
   (1) These abnormal RBCs are removed at a higher rate by the spleen.
   (2) Their survival is reduced when compared with normal RBCs.
   b. The patient often has an accompanying splenomegaly with cirrhosis, which further decreases RBC survival.
   c. The reticulocyte count is increased slightly, and the platelet count is normal or decreased.
   d. A differential diagnosis must distinguish this anemia from other macrocytic anemias. Patients who have anemia of liver disease usually have a normal WBC count and serum vitamin B12 and folate levels; patients who have megaloblastic anemias do not.

4. Anemias of endocrine disease can occur with hypothyroidism and androgen-deficient states.
   a. Anemia of hypothyroidism involves a mild-to-moderate anemia with a normal reticulocyte count.
      (1) Thyroid hormone regulates the cellular metabolic rate, and therefore, the tissue oxygen requirement.
      (2) With a decrease in thyroid hormone (i.e., hypothyroidism), there is a smaller tissue oxygen requirement, which is interpreted by the kidneys as a more than adequate oxygen tension. The net result is a decrease in the production of EPO.
      (3) This type of anemia often is a macrocytic/normochromic anemia but can also be a normocytic/normochromic anemia.
      (4) The anemia may be complicated by iron deficiency or folic acid or vitamin B12 deficiency, and the laboratory results may reflect these forms of anemia.
   b. A deficiency in testosterone secretion in men results in a decrease in RBC production and a decline of approximately 2 g/dL of Hb.

5. Myelophthisic anemia is an anemia associated with bone marrow infiltration and hyperproliferation by nonerythroid cells.
   a. A leukoerythroblastic reaction commonly accompanies the anemia.
      (1) Normoblasts of varying degrees of maturation are typically found on the blood smear with a reticulocytosis.
      (2) Increased leukocytes along with their immature forms are also found on the peripheral blood smear.
      (3) RBC morphology is normocytic/normochromic, but can be macrocytic.
      (4) Platelets are normal or decreased and often have abnormal forms.
   b. The usual causes of myelophthisic anemia include:
      (1) Metastatic carcinoma
      (2) Multiple myeloma
      (3) Leukemia
      (4) Lymphoma
      (5) Lipidoses or storage disease

6. Aplastic anemia is a marrow disorder characterized by a reduction in the number or function of multipotential stem cells with a resulting pancytopenia. This disorder is more commonly found in adults.
   a. The marrow is hypocellular with patchy areas of normal cellularity and increased fat cell infiltration.
   b. The diagnosis of severe aplastic anemia is made in pancytopenic patients when at least two of the following three peripheral blood values are found:
      (1) A WBC count lower than 500 cells/mm³
      (2) A platelet count lower than 20,000/mm³
      (3) A reticulocyte count lower than 1%
   c. Aplastic anemia can be acute and rapidly fatal or it can have a slow onset and a chronic course.
d. Most patients are initially seen with the following common clinical characteristics:
   (1) Bleeding as a result of thrombocytopenia
   (2) Increased susceptibility to infections due to leukopenia
   (3) All of the symptoms typical of anemia
   (4) The absence of splenomegaly
   (5) Iron overload from repeated transfusions

e. In 50% of patients, no specific agent can be correlated with the disease. Known causes of aplasia include:
   (1) Drugs (33%)
   (2) Chemicals or toxins (4%)
   (3) Infections and infectious hepatitis (4%)
   (4) Miscellaneous causes (59%)

f. The pathogenesis of aplastic anemia is thought to occur from either one or a combination of several of the following physiologic mechanisms:
   (1) Defective or insufficient multipotential colony-forming unit-spleen (CFU-S) stem cell population
   (2) Altered microenvironment that is unable to provide for normal differentiation and development of the stem cell compartment
   (3) Absent humoral and cellular stimulators for hematopoiesis
   (4) Excessive suppression of hematopoiesis by T lymphocytes or macrophages
   (5) Stem cells interacting among themselves with one clone inhibiting the growth of another
   (6) Immunologic mechanisms that suppress hematopoiesis

g. Treatment focuses on symptoms and finding the cause of suppression. Accepted courses of treatment include:
   (1) Blood transfusions
   (2) Stimulation of residual marrow with androgens or adrenocorticoids
   (3) Controlling infections with antibiotics
   (4) Controlling bleeding problems
   (5) Bone marrow transplantation for acceptable candidates younger than 40 years
   (6) EPO therapy to stimulate residual bone marrow

h. Laboratory values for a patient who has aplastic anemia include the following:
   (1) RBC morphology is normocytic/normochromic but often is macrocytic with a normal red cell distribution width (RDW)
   (2) Slight anisocytosis and poikilocytosis
   (3) Absence of circulating normoblasts in light of the degree of the anemia
   (4) An absolute leukopenia with a relative lymphocytosis
   (5) Thrombocytopenia
   (6) Elevated levels of leukocyte alkaline phosphatase (LAP)
   (7) Decreased marrow iron stores
   (8) High serum iron concentration
   (9) Very high plasma and urine EPO levels

i. Toxins. Aplastic anemia is often associated with toxic chemical or physical agents.
   (1) Toxic aplastic anemias are known to occur after exposure to mustard compounds, benzene, chemotherapy drugs (e.g., busulfan, urethan), antimetabolite drugs, or ionizing radiation.
   (2) Hyperresponsive aplastic anemia has been known to occur following treatment with certain classes of therapeutic drugs or chemicals that cause an autoimmune suppression of the bone marrow.
      (a) This effect has been noted with drugs such as antibiotics, anticonvulsants, analgesics, antithyroid drugs, antihistamines, and insecticides.
      (b) Chloramphenicol is the most common cause of drug-induced aplasia. Up to 50% of patients who receive this drug on a long-term basis will have a resulting mild pancytopenia that is reversible with drug withdrawal.
j. Aplastic anemia can occur secondary to other diseases or conditions, such as:
   (1) Infection, most often occurring as a sequel to infectious hepatitis
   (2) Paroxysmal nocturnal hemoglobinuria (PNH)
   (3) Pregnancy (usually remits after delivery)

k. Aplastic anemia (Fanconi’s anemia) refers to individuals with a genetic predisposition to bone marrow failure.
   (1) Detection occurs between the ages of 1 and 8 years.
   (2) Often, several family members are affected.
   (3) There is a high incidence of developmental abnormalities, which include some of the following symptoms:
      (a) Hyperpigmentation
      (b) Short stature
      (c) Hypogonadism
      (d) Malformation of the fingers and toes
      (e) Malformation of the organs
      (f) Abnormalities of the chromosome pattern of lymphocytes and marrow cells

7. Pure red cell aplasia is an unusual disease characterized by the selective depletion of only the erythroid bone marrow tissue.
   a. The disorder is a unipotential (CFU-E) stem cell defect that is believed to be related to an immunologic dysfunction.

b. Transitory arrest of erythropoiesis may occur in the course of a hemolytic anemia that is preceded by an infection.

c. Congenital RBC aplasia (Blackfan-Diamond anemia) is a rare disorder diagnosed between the ages of 1 and 6 years.
   (1) CFU-E and colony-forming burst (CFU-B) stem cells are decreased.
   (2) Patients have a severe normocytic or slightly macrocytic anemia and a low reticulocyte count.
   (3) Leukocytes and platelets are usually normal in numbers.
   (4) The marrow shows a reduction in all developing erythroid cells, except pronormoblasts.
   (5) Hemoglobin F (HbF) is elevated as high as 5% to 25%.
   (6) The RBC fetal antigen is present.

d. Acquired pure red cell aplasia is rare but occurs more in adults than in children.
   (1) Typical laboratory findings include a decreased reticulocyte count and marrow erythroid precursor depletion.
   (2) Up to 50% of patients have an associated cancer of the thymus (i.e., thymoma).
   (3) Remission of the anemia after surgical removal of the thymus occurs in 25% of patients.
   (4) Acquired red cell aplasia is thought to occur through the production of a cytotoxic autoantibody against erythroid precursors and a plasma inhibitor of heme synthesis.

8. Refractory anemia is an ill-defined group of chronic anemias occurring in persons older than 50 years.
   a. Laboratory features commonly include a normocytic or macrocytic anemia, decreased reticulocyte count, pancytopenia, and a hypercellular marrow with erythroid hypoplasia.
   b. Refractory anemia is now classified with the myeloproliferative disorders as one of five myelodysplastic syndromes.
   c. The anemia can develop into an acute leukemia with the presence of blast cells in the peripheral circulation.

C. Heme disorders represent a group of anemias that result from a defect in the synthesis of the heme ring in the mitochondria of developing normoblasts (Figure 4–3).
   a. Disorders of heme synthesis result in a microcytic/hypochromic anemia.
Heme synthesis begins with succinyl coenzyme A and terminates with the insertion of iron (Fe) into protoporphyrin IX. Enzyme deficiencies in the heme biosynthetic pathway are related to the various porphyrias (the name of the porphyria is placed by the enzyme that is deficient or dysfunctional). There is a buildup of the biosynthetic intermediates in the pathway prior to the enzyme deficiency, which results in an increase in these metabolites in the blood, feces, and urine. ALA = aminolevulinic acid.

Microcytosis is accompanied by an MCV lower than 80 fL (normal = 80–100 fL). Hypochromia is accompanied by an MCH < 25 pg (normal = 27–33 pg) and by an MCHC < 32 g/dL (normal = 32–36 g/dL).

Heme disorders can be caused by either enzyme deficiencies in the heme biosynthetic pathway (i.e., porphyrias), or by iron-related disorders.

2. Porphyrias are a group of inherited or acquired disorders that can occur from the deficiency of one or several biosynthetic enzymes. These disorders result in ineffective hematopoiesis and a hemolytic anemia.

a. The rate of biosynthesis of the heme ring is controlled to a large extent by the reaction rate of the beginning enzyme, δ-aminolevulinic acid (ALA) synthetase. The activity of this enzyme is reducible by some drugs and steroids.

b. Acute porphyrias are inherited enzyme deficiencies that begin with acute hemolytic episodes.
CHAPTER 4 Hematologic Disorders

(a) Clinical symptoms include:
   (i) Abdominal pain with vomiting
   (ii) Constipation
   (iii) Hypertension
   (iv) Peripheral neuritis
   (v) Behavioral changes and psychosis
(b) The common laboratory profile includes:
   (i) Slight elevations of bilirubin and alkaline phosphatase levels
   (ii) Increased urine ALA and porphobilinogen (PBG)
   (iii) Leukocytosis
(e) Enzyme defects. AIP is caused by decreased levels of uroporphyrinogen I and III synthetase, which results in increased production of ALA and PBG.

(2) Hereditary coproporphyria is a hereditary, autosomal dominant condition.
(a) Clinical symptoms. Patients are either asymptomatic, or they are initially seen with mild neurologic, abdominal, or psychiatric symptoms. Patients commonly have light-sensitive skin because of increased levels of PBG.
(b) The laboratory profile includes:
   (i) Increased coproporphyrinogen III in feces
   (ii) Intermittent increases of coproporphyrinogen, ALA, and PBG in urine.
(e) Enzyme defect. Patients have a deficiency in coproporphyrinogen decarboxylase activity.

(3) Variegate porphyria is a hereditary autosomal dominant condition that has a high incidence in the white population of South Africa.
(a) Clinical symptoms. Symptoms are similar to AIP with the additional symptom of cutaneous lesions, which are caused by highly photosensitive skin.
(b) Laboratory profile. There are increased levels of urinary ALA and PBG, but there is also an increase in the levels of porphyrin precursors that occur further along the pathway.
(e) Enzyme defect. Patients who have this disorder have a deficiency in heme synthetase (also known as ferrochelatase deficiency).

(5) Chronic porphyrias are more commonly associated with solar photosensitivity of the skin and include either genetic or acquired conditions.
(1) Congenital erythropoietic porphyria is rare and is inherited as an autosomal recessive disorder.
(a) Clinical symptoms of this disorder include:
   (i) Redness of the skin
   (ii) Hemolytic anemia
   (iii) Severe skin photosensitivity
   (iv) Splenomegaly
   (v) Often fatal early in life
(b) Laboratory profile. Patients often excrete red pigmented urine because of excessive excretion of coproporphyrin I and uroporphyrin I.
(e) Enzyme defect. Patients with this disorder show a deficiency in uroporphyrinogen III cosynthetase.

(2) Erythropoietic protoporphyria is an inherited disorder so named because the enzyme defects are localized in both the hepatic and erythropoietic cells.
(a) Clinical symptoms. This porphyria is a disease of early adulthood that exhibits mild skin photosensitivity.
(b) Laboratory profile. Patients who have this porphyria demonstrate an increased level of RBC photoporphyrin and elevated levels of fecal coproporphyrinogen and photoporphyrin.
(e) Enzyme defect causing this disorder is deficient activity of heme synthetase, which leads to over-reactive ALA synthetase.
Cutaneous hepatic porphyria (i.e., Cutanea Tarda) is an acquired disorder.

(a) **Clinical symptoms.** Patients are commonly first seen with skin lesions because of solar photosensitivity. Patients who have this porphyria have a medical history associated with liver disease, alcoholism, estrogen therapy, or ingestion of hexachlorobenzene.

(b) Characteristic laboratory profile results include an elevated urine uroporphyrin with the Type I isomers being excreted in greater amounts than the Type III isomers.

(c) **Enzyme defect.** Patients diagnosed with this disorder have reduced activity of the enzyme uroporphyrinogen III decarboxylase.

Lead intoxication is a commonly acquired porphyria that results from a block in several of the heme biosynthetic enzymes.

(a) **Clinical symptoms** of lead poisoning include:

(i) Abdominal pain
(ii) Constipation
(iii) Neuropathy
(iv) Absence of skin photosensitivity, ferrochelatase, and coproporphyrinogen oxidase

(b) The characteristic laboratory profile associated with lead toxicity includes:

(i) Elevated urinary ALA and coproporphyrin levels
(ii) Increased RBC photoporphyrin
(iii) Hypochromic RBCs with basophilic stippling due to increased erythropoiesis
(iv) Toxic granulation in neutrophils

(c) **Enzyme defect.** Lead inhibits several enzymes of the biosynthetic pathway. The most severely affected enzyme is ALA dehydrase.

3. **Iron metabolism-associated disorders** most commonly occur as a result of an iron deficiency but can result from a block of the enzyme that inserts iron (i.e., ferrochelatase) into the heme ring (Figure 4–3).

a. Iron and its metabolism are vital to the body because a Hb molecule is nonfunctional without iron, and two thirds or more of the total body iron is in the RBCs and their precursors.

1. Each milliliter of RBCs contains 1 mg of iron.

2. Storage iron is present in macrophages or normoblasts as ferritin (i.e., $\text{Fe}^{3+}$ plus apoferritin) or hemosiderin.

3. The majority of iron used in Hb synthesis is from the iron released and recycled by Hb that is degraded in macrophages and transported to normoblasts by plasma transferrin.

4. Only 0.9 to 1.3 mg of iron per day is lost from the body.

5. The small amount of iron lost from the body is maintained by dietary absorption, which amounts to approximately 1 mg/day.

(a) In men, dietary iron averages approximately 15 mg/day with only 6% intestinal absorption.

(b) In women, dietary iron averages approximately 11 mg/day with 12% intestinal absorption.

(c) Dietary absorption is increased in an iron deficiency state, but only to a maximum of 20%.

(d) Dietary iron is digested in the ferric form (i.e., $\text{Fe}^{3+}$) but is reduced to the ferrous form (i.e., $\text{Fe}^{2+}$) in the stomach by HCl and other reducing agents (e.g., food).

(e) The ferrous form is much more rapidly absorbed by the mucosal cells of the duodenum and upper jejunum than is the ferric form.

(f) Once in the mucosal cell, the ferrous form is oxidized again back to the ferric form and coupled to the protein apoferritin to form ferritin.

(g) Iron enters the circulation bound to transferrin.

(i) The normal serum iron concentration is 50 to 150 mg/dL.
The normal TIBC of transferrin is 250 to 400 mg/dL.

Transferrin is normally only 30% saturated.

Serum ferritin concentration is normally 15 to 200 ng/mL.

Normoblasts in the marrow have transferrin receptors and the capacity to extract iron from plasma transferrin.

The marrow receives only 5% of the total cardiac output, but it extracts 85% of the circulating iron.

Iron is also transferred to normoblasts via the “nurse” macrophage.

Iron deficiency anemia (IDA) is one of the most common forms of anemia in the United States.

IDA results when iron loss exceeds iron intake for a long time, and the body’s iron stores are depleted. Insufficient iron is available for normal heme production.

Iron deficiency develops when there is an increased need for iron, such as in rapid growth in infancy, childhood, or during pregnancy. IDA is the most common cause of anemia in children who are between the ages of 6 and 24 months.

Iron deficiency also develops when there is excessive chronic loss of blood.

In the adult man, even with no dietary iron intake, body iron stores of 1,000 mg would last for 3 or 4 years before iron depletion anemia would occur. Most cases of IDA in adult men, therefore, are attributed to chronic blood loss.

The development of IDA is seen to occur in three stages.

Iron depletion stage is the beginning of iron deficiency, when iron loss exceeds absorption. A negative iron balance develops, and iron is mobilized from iron stores. When this process begins, storage iron decreases, plasma ferritin decreases, gastrointestinal (GI) iron absorption increases to a limited extent, and the amount of plasma transferrin (i.e., TIBC) increases.

Iron deficient erythropoiesis stage occurs when the tissue iron stores are depleted. When transferrin saturation falls below 15%, the percent of marrow sideroblasts decreases, and as a result of insufficient iron for heme synthesis, the serum iron is decreased and levels of RBC photoporphyrin increase.

By the IDA stage, a clinical anemia becomes detectable first as a normocytic/normochromic anemia, then gradually progressing to a microcytic/hypochromic anemia.

Patients who have IDA are initially seen with the following clinical symptoms:

- Numbness and tingling of extremities
- Atrophy of the epithelium of the tongue with soreness
- Cracks or ulcers at the corners of the mouth
- Abnormal cravings for such things as dirt or ice (i.e., pica)
- Concave or spoon-shaped nails

Laboratory diagnosis of IDA demonstrates characteristic findings in the blood and bone marrow.

Laboratory findings from a peripheral blood sample include the following:

- Early stages of IDA may demonstrate normocytic/normochromic RBCs, but these eventually develop into microcytic/hypochromic RBCs with marked anisocytosis and slight poikilocytosis.
- Reticulocytes are decreased, except after iron therapy.
- The MCV is low, and the RDW is increased.
- The osmotic fragility is decreased.
- In most cases, the WBC count is normal, but granulocytes may tend toward a maturation shift to the right (i.e., hypersegmentation).
Table 4–2 Differential Diagnosis of the Microcytic/Hypochromic Anemias Using Iron-Related Parameters

<table>
<thead>
<tr>
<th>Disorder</th>
<th>Serum Iron</th>
<th>TIBC</th>
<th>% Sat</th>
<th>Ferritin</th>
<th>Bone Marrow Iron</th>
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<tbody>
<tr>
<td>Iron deficiency anemia</td>
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<td>↑</td>
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<td>Sideroblastic anemia</td>
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<tr>
<td>Thalassemia</td>
<td>N to ↑</td>
<td>N to ↓</td>
<td>N to ↑</td>
<td>N to ↑</td>
<td>N to ↑</td>
</tr>
</tbody>
</table>

N = normal; TIBC = total iron binding capacity; % Sat = percent transferrin iron saturation; ↑ = increased; ↓ = decreased.

(vi) Platelets are increased if the anemia is due to blood loss, or platelets may be decreased in advanced, severe anemic stages.
(vii) Serum iron is decreased (normal level is 50 to 150 mg/dL).
(viii) TIBC is increased (normal level is 250 to 400 mg/dL).
(ix) The percent saturation of TIBC (i.e., (serum iron ÷ TIBC) × 100) falls below 15% in IDA (normal is 20% to 55%).
(x) Serum ferritin is in equilibrium with tissue ferritin and is a good reflection of storage iron. Ferritin values usually decline below 12 ng/mL, which indicates low iron stores (normal range is 15 to 200 ng/mL).
(xi) Level of RBC intracellular photoporphyrin IX is increased in RBCs when TIBC percent saturation falls below 15%.

(b) The marrow of a patient who has IDA may have the following characteristics:
(i) Normoblasts are smaller than normal with frayed margins.
(ii) Storage iron is absent unless patient is receiving iron therapy.
(iii) Sideroblasts are decreased to lower than 20%.

(5) IDA must be differentially diagnosed from other microcytic/hypochromic anemias such as thalassemia, anemia of chronic disease, and sideroblastic anemia (Table 4–2).

c. Disorders of iron excess result from the slow accumulation of excess quantities of tissue iron stores. This can result from increased intestinal absorption, administration of transfused blood (i.e., transfusion hemosiderosis), or administration of iron-complex drugs or vitamins.

(1) General considerations
(a) Plasma iron increases to 200 mg/dL with 100% TIBC saturation.
(b) Macrophages are no longer able to store the extra iron once the percent TIBC saturation increases >50%.
(c) Excess iron slowly damages organs over a period of years.
(d) Hemochromatosis refers to the diseases and symptoms that arise from chronic iron overexposure.
(e) Young children are highly susceptible to acute iron poisoning due to accidentally swallowing iron pills or vitamins. Iron poisoning in children can be fatal with as little as a 50-mg dose.

(2) Primary familial hemochromatosis is a condition of iron excess that results from an abnormality in a gene on chromosome 6 that is closely linked with the human leukocyte antigen (HLA) locus.
(a) Iron overload stems from an overall increase in absorption by the intestinal mucosal cells.
(b) Patients chronically have an increased serum iron level, increased transferrin saturation, and iron-loading in macrophages and hepatocytes.
(3) Sideroblastic anemia is a disorder of iron excess that is associated with defective synthesis of heme because of multiple enzyme defects, and a resulting iron overload in the mitochondria of normoblasts. This anemia is now classified as a myelodysplastic syndrome.
(a) The patient’s RBC morphology is hypochromic and often microcytic.
(b) Patients are commonly seen with increased serum iron levels, decreased TIBC, and greatly increased transferrin saturation.

c) The marrow shows a greatly increased storage iron by an increase in sideroblasts >50% and an increase in siderotic granules per cell with granules surrounding the nucleus (i.e., ring sideroblasts).

(d) Several variations of sideroblastic anemia have been noted:

(i) A hereditary, X chromosome-linked type is found mostly in males and does not show up until adolescence.

(ii) Acquired idiopathic sideroblastic anemia can occur in either sex and has its onset in late adulthood. Some of the RBCs may be megaloblastic, and 10% of patients who have this type of anemia develop acute leukemia.

(iii) Pyridoxine-responsive (i.e., vitamin B6) sideroblastic anemia can be treated with high amounts of vitamin B6 to maintain normal Hb synthesis.

(iv) Drug-induced sideroblastic anemia has been known to occur in patients chronically exposed to antituberculosis drugs (e.g., Isoniazid), lead, chloramphenicol, or ethanol ingestion.

D. Globin chain disorders

1. General considerations. Globin dysfunction can occur from genetic mutations giving rise to either of the following two anemia classifications:

   a. Hemoglobinopathies represent the majority of abnormal Hb disorders. These Hb disorders result from a single amino-acid substitution in one of the polypeptide chains (i.e., α, β, γ, δ).

   b. Thalassemias are caused by an abnormal long or short polypeptide resulting from gene coding termination errors, frame-shift mutations, crossover in phase, deletion of codons, or fused hybrid chains. These gene coding abnormalities result in the deficiency or absence of one of the types of globin chains.

2. Hemoglobinopathies

   a. Nomenclature

      (1) The abnormal Hb disorder is most commonly represented by its letter abbreviation. For example, sickle cell anemia is denoted as Hb S, methemoglobinemia is written as Hb M.

      (2) Hb variants can also be written to describe their amino acid substitution (e.g., Hb S = α2β2 6 val).

   b. Sickle-cell disease (Hb SS) is a genetically homozygous condition that results in a serious chronic hemolytic anemia.

      (1) The anemia shows up at childhood and is often fatal by 30–40 years of age.

      (2) Hb S is found almost exclusively in the black population. Between 0.1% and 0.2% of American blacks are affected by this disorder.

      (3) Genetic alteration results in the glutamic amino acid in position 6 of the β-chain being replaced by a valine amino acid. This molecular alteration changes the Hb molecule’s overall charge and electrophoretic mobility.

      (4) Hb S is freely soluble in its fully oxygenated form.

         (a) In its deoxygenated form, polymerization of the abnormal Hb molecules occurs, which leads to the formation of intracellular crystals that deform the RBC to the sickle shape.

         (b) With homozygous Hb SS disease, sickling occurs even at physiologic oxygen tensions.

      (5) The rigidity of the RBC in the sickle shape is responsible for the trapping and hemolysis of the RBCs. This results in intravascular hemolysis, and the marrow becomes hyperplastic early in childhood, expanding the marrow space.

      (6) Clinical complications accompanying Hb SS disease are serious, resulting in systemic organ damage.
(a) Early in childhood, bilateral painful swelling of the hands and feet occur due to RBC sickling and capillary plugging (i.e., hand-foot syndrome).

(b) Splenic complications occur because of splenic blood sequestration and pooling of blood, which causes rapid spherocytosis and systemic hypovolemia. Patients can develop functional asplenia, which leads to an impaired immune system and renders the patient more susceptible to infections.

(c) Anoxic damage to the kidneys results from vascular plugging. Patients cannot produce a concentrated urine; they commonly are seen with hematuria (i.e., RBCs in the urine).

(d) Vaso-occlusive crises due to capillary occluding by sickle cells and loss of circulation to tissues intermittently affects patients with joint and abdominal pain.

(e) Aplastic crises can occur in the marrow because of systemic intravascular hemolysis.

(f) Leg ulcers are common because of capillary blocking and ischemic tissue.

(7) It is common for patients who have homozygous Hb SS to have the following hematologic profile:

(a) Normocytic/normochromic anemia

(b) Marked polychromasia on the blood smear

(c) Normoblasts found on the blood smear

(d) Numerous target cells and Howell-Jolly bodies because of asplenia

(e) Sickled RBCs found on the blood smear

(f) Decreased osmotic fragility test

(g) Neutrophilia and thrombocytosis

(8) The marrow aspirate shows normoblastic hyperplasia and increased iron storage.

(9) Common laboratory screening tests for Hb S include the following:

(a) Sickling of RBCs containing Hb S can be induced on a microscope slide with sodium metabisulfite.

(i) Metabisulfite is a reducing substance that enhances deoxygenation and sickling.

(ii) This test cannot differentiate homozygous Hb SS and heterozygous sickle cell trait (Hb AS).

(b) Solubility (i.e., turbidity) tests screen for Hb S with dithionite or toluene.

(i) The RBCs are first lysed, and Hb S is reduced by dithionite or toluene.

(ii) Reduced Hb S is insoluble in concentrated inorganic buffers, and the polymers of Hb S produce turbidity. The amount of turbidity is proportional to the amount of Hb S in the RBCs.

(10) A definitive diagnosis of Hb SS can be made with a hemoglobin electrophoresis at alkaline pH. Table 4–3 shows the electrophoresis results seen in homozygous Hb S. (Note: Abnormal hemoglobins Hb D and Hb G produce the same electrophoresis mobility, but the sickle-cell screening tests are negative.)

c. Hb AS is a heterozygous β-chain defect and the most common hemoglobinopathy in the United States. The genetic trait is present in 9% of American blacks and normally has no clinical signs or symptoms.

(1) With physiologic acidosis, hypoxia or high altitudes, respiratory tract infection, anesthesia, or congestive heart failure, sickling occurs with the same symptoms as seen in the homozygous form.

(2) Hb AS protects persons from the lethal effects of Falciparum malaria.

(3) The patient’s RBC count and morphology are normal, except for a few target cells.

(4) All sickle cell tests are positive.

(5) Heterozygous Hb AS produces the Hb electrophoresis results at an alkaline pH (Table 4–3).
### Table 4–3 Differential Diagnosis of the Globin-Chain Disorders Based on Hemoglobin Electrophoresis Results at Alkaline and Acid pH

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<th>α-Chain</th>
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<th>G</th>
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</tbody>
</table>

_a_ = origin or application of sample; _<–>_ = slightly increased; _+ _= 1%–25%; _++ _= 26%–50%; _+++ _= 51%–75%; _++++ _= 76%–100%; _− _= no data.

*Only shows up at birth.

### d. Hemoglobin C disease (Hb CC)

Hemoglobin C disease (Hb CC) is a genetically homozygous condition chiefly affecting blacks (0.02% in the United States) that presents as a moderate hemolytic anemia with splenomegaly, jaundice, and abdominal discomfort.

1. The _genetic alteration_ results in a _β-chain_ amino acid substitution of the glutamate at position 6 with a lysine amino acid.
2. Patients who have homozygous Hb CC are usually seen with the following hematologic profile:
   a. The anemia is normocytic/normochromic with a mixture of microcytes and spherocytes on the blood smear.
   b. A slight reticulocytosis is often noted.
   c. Numerous target cells (40%–90%) are found on the blood smear.
   d. The osmotic fragility test is biphasic.
   e. Hexagonal or rod-shaped crystals (i.e., Hb C crystals) may be found in RBCs on the blood smear. These crystals result from cellular dehydration of older RBCs, which therefore increases their rigidity, splenic trapping, and destruction.
3. Homozygous Hb CC produces the Hb electrophoresis results as shown in Table 4–3.

### e. The milder _heterozygous_ form (i.e., Hb AC), found in 2% to 3% of American blacks, is usually asymptomatic without anemia.
1. The most striking hematologic finding is a moderate number of target cells on the patient’s blood smear.
2. Heterozygous Hb AC produces the Hb electrophoresis results as seen in Table 4–3.

### f. Hb D disease and trait

Hb D disease and trait is most common in persons of Asian-Indian extraction.
1. The _genetic alteration_ in this disorder results in an amino acid substitution in the _β-chain(s)_ at position 121 of the glutamate with a glutamine amino acid.
The heterozygous trait is asymptomatic with a normal blood profile.

Hb D migrates on the electrophoretic gel with the Hb S fraction, but the sickle tests are negative. Hb D produces the Hb electrophoresis results as seen in Table 4–3.

Hb E disease and trait is found mainly in individuals of Asian origin.

Hb E produces the Hb electrophoresis results shown in Table 4–3.

Doubly heterozygous \( \beta \)-hemoglobinopathies are disorders in which the individual inherits a different abnormal \( \beta \)-chain gene from each parent.

Hb SC disease has an incidence similar to the homozygous Hb SS disease that occurs in American blacks.

The severity of the disease is intermediate between sickle cell trait and sickle-cell disease.

Onset is in childhood, but physical difficulties do not occur until the teenage years.

Basically, the symptoms are the same as Hb SS, but splenomegaly is more common in patients with Hb SC.

Patients are commonly seen with the following hematologic profile:

- Moderate-to-mild normocytic/normochromic anemia
- Moderate-to-severe anisocytosis and poikilocytosis
- Target cells can comprise up to 85% of the RBCs
- Sickle cells as well as Hb CC crystals on the blood smear
- Positive sickle cell tests

Hb SC produces the Hb electrophoresis pattern shown in Table 4–3.

Hb SD disease simulates but is less severe than Hb SS anemia. This disorder cannot be distinguished between Hb SC or sickle cell trait.

In some heterozygous hemoglobinopathies, the amino acid substitutions occur in the heme pocket where they either increase the stability of the Hb M or alter the affinity of the heme ring for oxygen.

Some abnormal hemoglobins are associated with cyanosis and Hb M.

The reduction to the ferrous heme and binding of oxygen is prevented by an amino acid substitution near the heme ring.

With \( \alpha \)-chain Hb M, cyanosis is present from birth. However, with \( \beta \)-chain Hb M, cyanosis does not occur until \( \beta \)-chain production reaches adult levels.

Some hemoglobinopathies result in increased oxygen-affinity and polycythemia.

Because of the high oxygen affinity, the tissue is hypoxic at any given oxygen tension. The result is increased EPO and hence, polycythemia.

Because the amino acid substitution is inside the molecule, the abnormality is indistinguishable from Hb A on Hb electrophoresis.

Example: Hemoglobin Chesapeake is an \( \alpha \)-chain abnormality that results in a mild polycythemia.

Some hemoglobins are associated with decreased oxygen affinity and cyanosis. An example of such an abnormal Hb is hemoglobin Kansas.

Unstable hemoglobins have an amino acid substitution at a place in the \( \alpha \) or \( \beta \) chain that affects the formation of the bonds between chains. The majority of these hemoglobins are \( \beta \)-chain substitutions.

Hb precipitates out in the circulating RBC and attaches to the RBC membrane.

Heinz bodies are Hb intracellular precipitates.

Intracellular precipitates render the cells inflexible and shorten RBC survival.

The majority of Heinz bodies are removed from the RBCs by the spleen.

Jaundice and splenomegaly are common, and patients may have a darkly pigmented urine.
Following splenectomy, Heinz bodies are numerous in the circulating RBCs.

Unstable hemoglobins produce the following Hb electrophoresis results at an alkaline pH:

(a) Approximately 25% of patients have a normal electrophoretic pattern
(b) Hb A2; increased in β-chain variants
(c) Hb F; increased to 10% to 15%

Unstable hemoglobins can also be detected in the laboratory with the heat instability test and the isopropanol precipitation test.

3. Thalassemias

a. Background

(1) Thalassemias are disorders of Hb synthesis that occur mainly in persons of Mediterranean, African, and Asian ancestry.
(2) The dysfunction is a diminished rate of synthesis of certain globin chains, but the chain formed in most cases is of normal amino acid sequence.
(3) Total lack of α- or β-globin production is known as thalassemia major. Production at a decreased rate is known as thalassemia minor.
(4) α-Thalassemia is associated with a decrease in the production of α-chains. β-Thalassemia is associated with a decrease in the production of β-chains.

b. Globin-chain molecular defects and nomenclature includes the following:

(1) β°-Thalassemia denotes a condition in which β-chain synthesis is absent. The gene is not deleted, but the messenger ribonucleic acid (mRNA) is absent or nonfunctioning.
(2) β+-Thalassemia is a condition in which β-chain synthesis is only reduced. Such disorders result from defects in transcription and processing of mRNA.
(3) δβ-Thalassemia is caused by gene deletions involving both the δ and β genes.
(4) γδβ-Thalassemia is caused by a long deletion including the γ-genes and δ-genes that stops short of the β-gene, but also reduces the output of β-chains.
(5) Lepore thalassemia shows normal α-genes. Abnormal δβ-genes caused by δβ-fusion of genes results from gene crossover.
(6) α-Thalassemias are generally caused by gene deletions of various lengths and can also be αα or αα-.
(7) Hb constant spring is caused by an abnormal termination codon in an α-chain. This results in an elongated α-chain with 31 extra amino acids and slowed or reduced α-globin synthesis.

c. Homozygous β-Thalassemia (thalassemia major) results from either a decrease or an absence in β-chain production by both gene alleles.

(1) With a decrease in β-chain production, γ-chain production is high, which results in increased Hb F.
(2) There is an excess of α-chains due to a lack of matching β-chains.
   (a) Unstable tetramers of α-chains (i.e., αα) precipitate out of solution in the normoblast or RBC.
   (b) These α-chain precipitates adhere to the inner membrane and damage the cell, which results in ineffective erythropoiesis and a severe hemolytic anemia.
(3) Clinical symptoms of β-Thalassemia major include:
   (a) Jaundice and splenomegaly early in childhood
   (b) Prominent frontal bones (i.e., cheek, jaws)
   (c) Chronic marrow hyperplasia resulting in a thinned cortex of the long bones
   (d) Stunted growth and delayed puberty
   (e) Hemochromatosis from regular transfusions
   (f) Cardiac failure (i.e., major cause of death) due to myocardial siderosis by 30 years of age
Patients who have homozygous β-Thalassemia commonly present with the following hematologic profile:
(a) Unlike most hemolytic anemias, β-Thalassemia is morphologically a microcytic/hypochromic anemia caused by a defect in either the rate of Hb synthesis or in the lack of synthesis.
(b) Peripheral blood smears show extreme poikilocytosis and anisocytosis in RBCs, as well as such morphologic abnormalities as target cells, ovalocytes, Howell-Jolly bodies, normoblasts, siderocytes, and Cabot’s rings.
(c) The reticulocyte count is less elevated than what would be expected for the degree of anemia because of the destruction of erythroid precursors in the marrow.
(d) The osmotic fragility test is decreased.
(e) Serum iron is increased.
(f) The indirect bilirubin level is increased.
(g) The MCV is decreased with an increased RDW.

Marrow aspirate reveals normoblastic hyperplasia, increased storage iron, and sideroblasts.

Homozygous β-Thalassemia can exist in three genetic forms, each producing a different electrophoresis pattern (Table 4–3).
(a) β+ Thalassemia represents a partial decrease in β-chain production. Persons of Mediterranean descent with this form of thalassemia have severe clinical symptoms. They demonstrate the electrophoretic pattern as seen in Table 4–3.
(b) β- Thalassemia is a less clinically severe disorder that occurs in persons of African descent. These patients show the electrophoretic pattern as seen in Table 4–3.

Heterozygous β-Thalassemia (i.e., thalassemia minor) results from the absence or decrease in β-chain production at one gene allele.

Patients may be seen with the following clinical symptoms:
(a) The degree of anemia may vary from a severe microcytic/hypochromic anemia to normal clinical findings.
(b) A moderate-to-severe anemia is more common in individuals of Mediterranean descent.
(c) Patients who have more severe disease have a slight hemolytic jaundice and splenomegaly.

Patients who have heterozygous β-Thalassemia are commonly seen with the following hematologic profile:
(a) Characteristically, the RBC count is increased, and the Hb and Hct are reduced.
(b) The RBC indices are commonly below normal, and the RDW is increased.
(c) RBC morphology on the peripheral blood smear shows moderate poikilocytosis, target cells, and basophilic stippling.
(d) Osmotic fragility is decreased.
(e) Serum iron levels are normal to high.

Marrow often demonstrates the same characteristics as homozygous β-Thalassemia.

Thalassemia minor produces the Hb electrophoresis results shown in Table 4–3.

δ-β-Thalassemias produce clinical symptoms similar to β-Thalassemias.
(a) Homozygotes have intermediate thalassemia symptoms, and their electrophoretic pattern shows an absence of Hb A and Hb A2.
(b) Heterozygotes clinically have thalassemia minor symptoms and demonstrate 5% to 20% Hb F and normal Hb A2 on their electrophoretic pattern.

Hb Lepore syndromes produce an abnormal Hb that has a normal α-chain combined with a fused δβ-chain.
(a) Because the δβ-chain is synthesized at a slower rate, it results in a microcytic/hypochromic anemia and resembles a thalassemia.
CHAPTER 4 Hematologic Disorders

(b) Hb Lepore migrates with the Hb S band on electrophoresis gel at an alkaline pH.

e. Double heterozygotes for β-Thalassemia and β-hemoglobinopathies occur when an individual inherits a thalassemia gene from one parent and a hemoglobinopathy gene from the other parent.

(1) Hb A levels are always less than levels of the variant Hb.

(2) Sickle cell thalassemia (i.e., Hb S β-Thalassemia) symptomatically differs from Hb SS in that patients have splenomegaly from childhood into adulthood.

(a) This disorder is clinically similar to sickle-cell disease, except that the MCV and MCH are lower, and Hb A2 is increased to greater levels than that seen in Hb SS.

(b) Patients are commonly seen with the following distinguishing hematologic characteristics:

(i) Marked microcytosis

(ii) Variable hypochromia

(iii) Many target cells

(iv) Rare sickle cells

(v) Low MCV and MCH

(vi) Positive sickle cell tests

c. Hb S β-Thalassemia demonstrates the following electrophoresis pattern:

(i) Hb A: none

(ii) Hb S: 75% to 90%

(iii) Hb F: 5% to 20%

(iv) Hb A2: >4.5%

d. Hb S β+-Thalassemia demonstrates the following electrophoresis pattern:

(i) Hb A: 15% to 30%

(ii) Hb S: >50%

(iii) Hb F: 1% to 20%

(iv) Hb A2: increased >4.5%

(3) Hb C and Thalassemia occurs mainly as a mild anemia in persons of African descent and as a severe anemia in a smaller percentage of persons of Mediterranean descent.

(a) Hb C β-Thalassemia demonstrates the following electrophoresis pattern:

(i) Hb C: 90% to 95%

(ii) Hb F: 5% to 10%

(iii) Hb A: none

(b) Hb C β+-Thalassemia demonstrates the following electrophoresis pattern:

(i) Hb A: 20% to 30%

(ii) Hb C: 70% to 80%

(iii) Hb F: normal

(iv) Hb A2: masked on electrophoresis by Hb C

(4) Hb E β-Thalassemia is a Southeast Asian disease resembling thalassemia major and demonstrates the following characteristic electrophoresis pattern:

(a) Hb E: 15% to 95%

(b) Hb F: 5% to 85%

(c) Hb A: none

f. α-Thalassemias result from a partial or total decrease in the production of α-chains.

(1) Although there are two β-globin genes per diploid genotype, there are four α-globin genes per diploid genotype. These are normally designated as α2α2.

(a) Mild α-Thalassemia (i.e., αα+-Thalassemia) is a deletion of only one gene and is represented as αα/αα.
Severe α-thalassemia (i.e., α°-Thalassemia) is a deletion of two α-globin genes, and is represented as –/αα.

Hydrops fetalis with Hb Bart’s is the most severe form of α-thalassemia. Individuals who have this disorder have a diploid genotype of –/–, which represents the absence of all α-chains.

The absence of all α-chains is incompatible with life, and infants who have this disorder are stillborn or die soon after birth.

In the absence of α-chains, γ-chains and β-chains form tetramers known as Hb Bart’s and Hb H, respectively. Both of these tetramer forms migrate faster than do Hb A on electrophoresis gel.

This disorder demonstrates the following characteristic electrophoretic pattern:

(i) Hb A, Hb F, and Hb A2: none
(ii) Hb Bart’s: >80%

Hb H disease (i.e., α-thalassemia major) is a thalassemia in which three of the four α-globin genes are absent (i.e., –/αα).

Clinically, patients who have this disorder have a chronic hemolytic anemia that resembles an intermediate thalassemia.

Patients are commonly seen with the following distinguishing hematologic characteristics:

(i) Decreased MCV and MCH
(ii) Hypochromia and target cells on the blood smear
(iii) Moderate anisocytosis and poikilocytosis on the smear
(iv) A reticulocyte count of 4% to 5%
(v) Moderate Heinz bodies, which are Hb H precipitates

This disorder demonstrates the characteristic electrophoretic pattern shown in Table 4–3.

α-thalassemia minor is clinically mild and resembles β-thalassemia minor. Genetically, patients are lacking two of the four α-globin genes (i.e., –/αα or –α/αα).

Patients have a mild anemia, microcytosis, and normal serum iron.

This disorder demonstrates the following characteristic electrophoretic pattern:

(i) Diagnosis is made by finding 5% to 6% of Hb Bart’s in cord blood.
(ii) Adults show no evidence of an Hb imbalance.

Silent carrier of α-thalassemia (i.e., heterozygous α+/α-thalassemia) represents a disorder that has only one defective α-globin gene (i.e., –α/αα) and is not associated with any hematologic abnormalities.

Hemoglobin constant spring (Hb CS) is an α-chain variant with 31 extra amino acids.

α-Chains are functionally normal but synthesized more slowly, and therefore present a clinical picture of thalassemia.

Hb CS migrates slower than Hb A2 at an alkaline pH.

This disorder demonstrates the following characteristic electrophoretic pattern:

(i) Hb CS: 5% to 6%
(ii) Hb A2: normal
(iii) Hb Bart’s: trace
(iv) Hb A: major fraction

E. Deoxyribonucleic acid (DNA) disorders. Megaloblastosis is almost always caused by deficiencies of vitamin B₁₂ or folic acid.

1. Megaloblastic anemia as a macrocytic condition. Macrocytosis caused by megaloblastic anemia must be differentiated from macrocytosis with a normoblastic marrow (Figure 4–4).

2. Macrocytic anemia with a megaloblastic marrow can result in ineffective hematopoiesis.
Figure 4–4 Algorithm for the differential diagnosis of the megaloblastic anemias from other macrocytic anemias. 

- **Figure 4–4** Algorithm for the differential diagnosis of the megaloblastic anemias from other macrocytic anemias. MCH = mean corpuscular hemoglobin; MCHC = mean corpuscular hemoglobin concentration; MCV = mean cell volume; RBC = red blood cell; RDW = red cell distribution width.

- a. The laboratory profile of a patient who has megaloblastic anemia is given in Table 4–4.

- b. The marrow is characterized by enlargement of precursor cells. Marrow is hypercellular because of increased EPO stimulation; however, erythropoiesis is ineffective (Figure 4–5).

- 3. Vitamin B₁₂ (i.e., cyanocobalamin)
  - a. Metabolism occurs in the small intestine. Dietary B₁₂ is released from digestion of animal proteins in meats and is bound by gastric intrinsic factor (IF).
  - b. Normal serum vitamin B₁₂ values range from 200 to 900 ng/L. If vitamin B₁₂ intake is stopped, total body stores of 2 to 5 mg last for several years before a megaloblastic anemia results.
  - c. Vitamin B₁₂ function is related to DNA synthesis because vitamin B₁₂ is a vital cofactor in the conversion of methyl tetrahydrofolate (i.e., folic acid) to tetrahydrofolate. This substrate is an important cofactor needed for the production of thymidine, which is a DNA base.
  - d. Common cause of vitamin B₁₂ deficiency. Inadequate dietary intake of vitamin B₁₂ is extremely rare in the United States; it is usually seen in strict vegetarians. The most common cause of vitamin B₁₂ deficiency is defective production of IF.
### Table 4-4 Laboratory Profiles of DNA Disorders

<table>
<thead>
<tr>
<th>Disorder</th>
<th>Characteristics</th>
<th>Laboratory Profile</th>
</tr>
</thead>
<tbody>
<tr>
<td>Megaloblastic anemia</td>
<td>Marrow has enlarged precursor cells. Blood smear RBC morphology shows macrocytes mixed with microcytes, moderate anisocytosis and poikilocytosis, basophilic stippling, Howell-Jolly bodies, Cabot’s rings, and a few NRBCs.</td>
<td>Pancytopenia Low RBC count Low hemoglobin Low hematocrit MCV &gt; 100 fL Increased to normal MCH Leukopenia with hypersegmentation of neutrophils Mild thrombocytopenia Increased serum iron</td>
</tr>
<tr>
<td>Vitamin B&lt;sub&gt;12&lt;/sub&gt; deficiency</td>
<td>Same characteristics as megaloblastic anemia Serum vitamin B&lt;sub&gt;12&lt;/sub&gt; assay indicates value &lt; 100 ng/L (normal = 200–900 ng/L) Increased urinary methylmalonic acid Shilling test can indicate an intrinsic factor dysfunction or pernicious anemia</td>
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</tr>
<tr>
<td>Folic acid deficiency</td>
<td>Leukopenia and thrombocytopenia are less common than with vitamin B&lt;sub&gt;12&lt;/sub&gt; deficiency Serum folate &lt; 3 mcg/L (normal = 5–21 mcg/L) RBC folate &lt; 150 mcg/L (normal = 150–600 mcg/L) Urinary formiminoglutamic acid level is increased</td>
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</tr>
</tbody>
</table>

DNA = deoxyribonucleic acid; MCH = mean corpuscular hemoglobin; MCV = mean corpuscular volume; NRBC = nucleated red blood cells; RBC = red blood cell.

1. Pernicious anemia (PA) is a “conditioned” nutritional deficiency of B<sub>12</sub> caused by failure of the gastric mucosa to secrete IF.
2. PA is an inherited disorder, most commonly occurring in persons older than 40 years (Table 4–4). Two types of autoantibodies have been identified in PA patients:
   a. An autoantibody that is directed against the parietal cells is found in 85% to 95% of PA patients.
   b. Autoantibodies can also develop that are directed against IF or the IF-vitamin B<sub>12</sub> complex.
3. Other causes of PA include the following:
   a. Gastrectomy (i.e., the surgical removal of part or all of the stomach) removes the source of IF and results in megaloblastic anemia if vitamin B<sub>12</sub> supplements are not provided.
   b. Defective absorption of vitamin B<sub>12</sub> into the intestinal mucosal cell can result in a secondary vitamin B<sub>12</sub> deficiency, even if dietary intake is normal.
   c. Lack of availability of vitamin B<sub>12</sub> in the small intestine can result from competition for dietary B<sub>12</sub>.
   i. In some countries, infestation with the fish tapeworm Diphyllobothrium latum is common and results in vitamin B<sub>12</sub> deficiency because the worm competes for the available dietary vitamin B<sub>12</sub>.
   ii. Bacterial infestation in a blind loop of the intestine also uses ingested vitamin B<sub>12</sub>.
4. Diagnosis of vitamin B<sub>12</sub> deficiency is based upon clinical symptoms, as well as laboratory results (Table 4–4).
5. Folic acid
   a. Sources. Folate is primarily acquired from the diet in such foods as eggs, milk, leafy vegetables, yeast, liver, and fruits. A smaller percentage is formed by intestinal flora.
   b. The minimum daily dietary requirement has been set at 50 mg. Dietary ingestion just barely meets the minimum daily requirement. The body’s reserve lasts for only 3 months.
c. **Storage.** Liver tissue is the main storage site of folic acid.

d. **Normal serum reference values are** 5 to 21 mcg/L, and 150 to 600 mcg/L for RBC folate.

e. **Deficiency.** Clinically, symptoms from inadequate dietary folate can occur within weeks, as compared with years for a vitamin B\(_12\) deficiency. Leukopenia and thrombocytopenia are less common with folate deficiency than vitamin B\(_12\) deficiency, but the symptoms and the hematologic profile are generally the same (Table 4–4). Unlike vitamin B\(_12\) deficiency, a megaloblastic anemia caused by folate deficiency is most commonly due to insufficient dietary intake. A woman’s demand for folate increases during pregnancy, and pregnant women should receive folate supplements of approximately 500 μg/day.

f. **Causes of deficiency**

(1) **Liver disease associated with alcoholism** results in a dietary folate deficiency. A differential diagnosis is necessary to distinguish this condition from an anemia of liver disease, which has a normal folate but is macrocytic with a normoblastic marrow.

(2) **Defective absorption of folate in the small intestine** can result from malabsorption syndromes, such as nontropical sprue, intestinal blind-loop syndrome, and adult celiac disease.
### Table 4–5 Characteristics and Laboratory Profiles of RBC Membrane Disorders

<table>
<thead>
<tr>
<th>Disorder</th>
<th>Characteristics</th>
<th>Laboratory Profile</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemolysis</td>
<td>Normocytic/normochromic anemia</td>
<td>Increased serum bilirubin</td>
</tr>
<tr>
<td></td>
<td>Polychromasia on blood smear</td>
<td>Decreased serum haptoglobin</td>
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<tr>
<td></td>
<td>Spherocytes</td>
<td>Bone marrow shows normoblastic hyperplasia</td>
</tr>
<tr>
<td></td>
<td>Polychromasia on blood smear</td>
<td>Storage iron and sideroblasts increased</td>
</tr>
<tr>
<td></td>
<td>Other RBC abnormalities</td>
<td>Possible aplastic crisis</td>
</tr>
<tr>
<td></td>
<td>Leukocytosis with maturation shift to left if hemolysis is acute</td>
<td>reticulocytopenia erythroid hypoplasia</td>
</tr>
<tr>
<td></td>
<td>Spherocytosis</td>
<td>Possible hemolytic anemia</td>
</tr>
<tr>
<td>Hereditary spherocytosis</td>
<td>Spherocytic RBCs intrinsically defective smaller diameter no central pallor</td>
<td>Osmotic fragility test increased</td>
</tr>
<tr>
<td></td>
<td>Defect in spectrin</td>
<td>MCHC increased</td>
</tr>
<tr>
<td>Hereditary elliptocytosis</td>
<td>Splenomegaly</td>
<td>Osmotic fragility test decreased</td>
</tr>
<tr>
<td></td>
<td>Few Spherocytes</td>
<td>Abnormal autohemolysis test results</td>
</tr>
<tr>
<td>Hereditary pyropoikilocytosis</td>
<td>Microcytosis Marked poikilocytosis and RBC fragmentation Defective spectrin function</td>
<td>Abnormal RBC fragmentation at 45°C–46°C.</td>
</tr>
<tr>
<td>Paroxysmal nocturnal hemoglobinuria</td>
<td>Chronic normocytic/ normochromic anemia Microcytic/hypochromic</td>
<td>Osmotic fragility test normal</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Increased autohemolysis</td>
</tr>
</tbody>
</table>

MCHC = mean corpuscular hemoglobin concentration, RBCs = red blood cells.

(3) Inadequate utilization of folate in the body can be blocked with chemotherapy drugs (e.g., methotrexate), which are folic acid antagonists. In addition to inhibiting tumor growth, chemotherapeutic drugs also produce a megaloblastic anemia.

g. Diagnosis of a folate deficiency is based upon the common clinical symptoms, as well as laboratory results (Table 4–4).

### F. Survival disorders: hemolytic anemias

1. Common laboratory characteristics of hemolysis. Hemolysis occurs whenever there is increased RBC destruction and shortened cell survival. In general, all hemolytic anemias can be categorized into one of two groups (Figure 4–5).

   a. Intrinsic hemolytic anemias are usually hereditary and occur from defects in the RBC membrane, metabolism, or the Hb molecule.

   b. Extrinsic hemolytic anemias represent the RBC survival disorders that are acquired and occur secondary to a primary condition or stimulus.

2. Hemolysis caused by RBC membrane disorders. The common clinical and laboratory characteristics of these disorders are given in Table 4–5.

   a. Hereditary spherocytosis (HS) is an autosomal dominant condition that exhibits a moderate-to-severe anemia.

      (1) Cause. Splenomegaly is the cause of the shortened RBC survival. The chronic hemolytic anemia is often corrected by splenectomy. HS is caused by a defect in spectrin, which is an RBC peripheral protein.

      (2) Laboratory tests useful in the diagnosis of HS include:

      | a) Blood smear with spherocytes |
      | b) Osmotic fragility test      |
      | c) Autohemolysis test          |

   b. Hereditary elliptocytosis (HE) is an autosomal dominant condition that results in a defect of spectrin assembly during the formation of the RBC skeletal lattice.
Elliptocytes are abundant and often represent more than 25% of the RBC population.

Of all patients who have HE, 90% are nonanemic. The other 10% of patients have a mild-to-moderate hemolytic anemia.

c. Hereditary pyropoikilocytosis (HPP) is an autosomal recessive, severe hemolytic anemia that occurs rarely in blacks. HPP is also thought to involve defective spectrin function.

d. Hereditary stomatocytosis results in a mild-to-moderate hemolytic anemia. Stomatocytes are RBCs that, on an air-dried blood film, have a slit-shaped central pallor rather than a circular central pallor. Stomatocyte forms are not as flexible as the normal biconcave disk-shaped RBC, and they have a shortened survival time.

e. Paroxysmal nocturnal hemoglobinuria (PNH) occurs more frequently in young adults as an acquired intrinsic defect of the RBC membrane. The defect renders the RBCs hypersensitive to complement C3 binding, and therefore, hemolysis. PNH is characterized by chronic intravascular hemolysis with or without hemoglobinuria.

Hemoiderinuria is also present. If excessive, it can lead to a serious iron loss.

Two or three different populations of RBCs with different degrees of complement sensitivity are present.

Platelets and neutrophils may also be hypersensitive to complement, and therefore low in count.

Laboratory tests useful in the diagnosis of PNH include the following:

- The sucrose hemolysis test (i.e., sugar-water test) is based on the principle that sucrose provides a medium of low ionic strength and promotes the binding of complement to RBCs. Patients who have PNH demonstrate a higher degree of hemolysis with this procedure.

- The acidified serum test (i.e., Ham test) is based on the principle that acidified serum activates complement by the alternate pathway. Patients who have PNH demonstrate a higher degree of hemolysis with this procedure.

3. Hemolysis caused by RBC metabolic disorders.

a. Glucose-6-phosphate dehydrogenase (G6PD) deficiencies are inherited as a sex-linked trait. Anemia caused by a G6PD deficiency is found in all races, but the highest incidence is among African and Mediterranean cultures.

Two genetic isoenzyme variations of G6PD have been identified. These subtypes are: Type A and Type B, depending on their electrophoretic mobility.

- Type A. Approximately 10% of blacks who have Type A isoenzyme have an inherited deficiency that results in only 10% to 15% G6PD enzyme activity. These individuals have clinically mild conditions but can be affected by oxidants to produce a hemolytic crisis.

- Type B. Persons of Mediterranean descent who have a G6PD deficiency more commonly have a Type B deficiency. Patients who have a Type B deficiency have an enzyme activity of only 1%. These patients are more susceptible to severe oxidant hemolysis than are Type A individuals.

- Favism. A subgroup of G6PD-deficient patients have severe life-threatening hemolysis that occurs within hours after eating fava beans.

Cause. Nicotinamide-adenine dinucleotide phosphate reduction (NADPH) in the RBC is linked to glutathione (GSH) reduction. GSH is a vital reducer of oxidants, such as hydrogen peroxide. This metabolic pathway preserves vital enzymes and Hb. A deficiency of G6PD limits the regeneration of NADPH, and therefore GSH. Lack of GSH renders the RBC vulnerable to the oxidative degeneration of Hb.

G6PD is normally highest in young RBCs and decreases as the cell ages; therefore, the older RBCs are preferentially destroyed.
Table 4–6  Laboratory Tests Useful for Diagnosing Hemolysis Caused by Metabolic Disorders

<table>
<thead>
<tr>
<th>Deficiency</th>
<th>Laboratory Test</th>
<th>Principle</th>
</tr>
</thead>
<tbody>
<tr>
<td>G6PD deficiency</td>
<td>Methyl violet or crystal violet stains</td>
<td>Heinz bodies stain</td>
</tr>
<tr>
<td></td>
<td>Dye reduction test</td>
<td>G6P, NADP, and brilliant cresyl blue dye mixture is incubated; if G6PD is present in hemolysate, NADP will be oxidized, and blue dye will change color</td>
</tr>
<tr>
<td></td>
<td>Ascorbate cyanide test</td>
<td>Hemoglobin is oxidized to Hb M by ascorbate more rapidly in G6PD-deficient patients because they lack GSH</td>
</tr>
<tr>
<td></td>
<td>Fluorescent spot test</td>
<td>A drop of G6P and NADP is placed on filter paper with patient’s hemolysate; if G6PD is present, NADP is oxidized to NADPH, which fluoresces with UV light</td>
</tr>
<tr>
<td></td>
<td>Quantitative assay of G6PD</td>
<td>Rate of reduction of NADP to NADPH can be measured spectrophotometrically at 340 nm</td>
</tr>
<tr>
<td>PK deficiency</td>
<td>Fluorescent spot test</td>
<td>RBCs with PK activity reduce any NADH to nonfluorescent NAD; if fluorescence persists, PK deficiency is indicated</td>
</tr>
<tr>
<td></td>
<td>Quantitative assay for PK</td>
<td>Same principle as for G6PD; rate of decrease is measured at 340 nm</td>
</tr>
</tbody>
</table>

G6P = glucose-6-phosphate; G6PD = glucose-6-phosphate dehydrogenase; GSH = reduced glutathione; Hb M = hemoglobin M; NAD = nicotinamide adenine dinucleotide; NADH = reduced nicotinamide adenine dinucleotide; NADP = nicotinamide adenine dinucleotide phosphate; NADPH = reduced nicotinamide adenine dinucleotide phosphate; PK = pyruvate kinase; RBCs = red blood cells; UV = ultraviolet.

(b) Oxidized Hb denatures and precipitates intracellularly as Heinz bodies, which adhere to the membrane and thus cause rigidity, a tendency to lyse, and splenic trapping.

(e) A patient’s susceptibility to hemolytic crisis can greatly increase with an illness or exposure to drugs that have oxidant properties.

(d) Patients who have G6PD deficiency have a moderate-to-severe hemolytic anemia and a high incidence of Heinz bodies in their RBCs. Laboratory tests useful in the diagnosis of G6PD deficiency are given in Table 4–6.

b. Pyruvate kinase (PK) deficiency is a rare, autosomal recessive condition.

(1) Cause. The enzyme PK catalyzes the conversion of phosphophenylpyruvate to pyruvate in the Embden-Meyerhof pathway with the production of adenosine triphosphate (ATP). As a result of the decrease in ATP needed to maintain RBC membrane stability and flexibility, this deficiency results in a mild-to-moderate hemolytic anemia. The RBC loses its flexibility, and splenic trapping and removal of the RBC ensues. Patients who have PK deficiency may not have any observable RBC abnormalities until after splenectomy.

(2) Laboratory tests useful in the diagnosis of PK deficiency are given in Table 4–6.

4. Acquired extrinsic hemolysis
a. Causes. Physical forces can destroy the shape of the normal RBC and result in fragmentation.

(1) Heat from extensive burns results in a hemolytic anemia with RBC fragments.

(2) Cardiovascular disease and prostheses can produce hemolysis due to mechanical damage of RBCs.

b. Microangiopathic hemolytic anemia with moderate RBC fragmentation can result as a secondary manifestation of the following conditions:

(1) Chronic hypertension
(2) Thrombotic thrombocytopenic purpura
(3) Disseminated carcinoma
(4) DIC

c. Immune hemolytic anemias result from immunoglobulin binding to the RBC membrane and splenic removal of the cells.
(1) Autoimmune hemolytic anemia (AIHA) presents a hematologic picture of a hemolytic anemia with a positive direct antiglobulin test (i.e., Coombs’ test).
(a) AIHA can be associated with warm antibodies. These immunoglobulins are usually immunoglobulin G (IgG) autoantibodies directed against Rh antigens that demonstrate maximum RBC binding at 37°C.
(b) AIHA associated with cold antibodies are most often immunoglobulin M (IgM) autoantibodies that react most strongly at or below 25°C. (i) Cold hemagglutinin disease involves complement-fixing autoantibodies against the I antigen. (ii) Paroxysmal cold hemoglobinuria is most often caused by a complement-fixing IgG autoantibody directed against the P antigen.

(2) Isoimmune hemolytic anemia is caused by an immune response to a foreign antigen. An example is hemolytic disease of the newborn (HDN), which is caused by Rh or ABO incompatibility between mother and fetus.

(3) Drug-induced immune hemolytic anemias are caused by the adsorption of drug-stimulated immune complexes to RBC membranes.
(a) The drug-induced antibody fixes complement that binds to the RBC membranes, which results in lysis.
(b) Drugs such as penicillin or cephalosporin can act as haptons, inducing an immune response when they attach to the RBC membrane.

5. Anemia of blood loss
a. Acute posthemorrhagic anemia, caused by a sudden blood loss, first results in early hypovolemia without any signs of anemia.
(1) After approximately 24 hours, blood volume is returned to normal to maintain blood pressure. Fluid from tissues move into the circulation, increasing the plasma volume. When a systemic fluid shift occurs, anemia becomes evident.
(2) Hematologic changes seen with acute blood loss include the following:
(a) The earliest change is a brief thrombocytopenia, which soon returns to normal.
(b) A moderate leukocytosis with a shift to the left occurs for the first few days after hemorrhage.
(e) Hb and Hct do not decline until plasma volume is increased by inward movement of fluid.
(d) After 2 to 3 days, normocytic-normochromic anemia occurs with minimum anisocytosis and poikilocytosis.
(e) Increased erythropoietin release results in a reticulocytosis after 3 to 5 days and peaks at approximately 10 days after hemorrhage. With reticulocytosis, the anemia may be macrocytic with polychromasia caused by shift reticulocytosis.

b. Chronic posthemorrhagic anemia is caused by slow, sustained blood loss (e.g., GI bleeding).
(1) The reticulocyte count may be normal or only slightly increased.
(2) The anemia that develops is one of iron deficiency and presents as a microcytic-hypochromic anemia.
(3) The WBC count is normal or slightly decreased.
(4) Thrombocytosis is common until iron deficiency is severe.

IV. LEUKOCYTE DISORDERS
A. Blood film white cell morphology and differential
1. Staining the blood film
a. Blood dyes are of two general types: methylene blue and eosin. Cell structures are stained by either one or a combination of both dyes.
(1) Methylene blue is known as a basic stain.
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(a) WBC nuclei, cytoplasmic RNA, and platelets are stained by basic stains.
(b) Staining of cell structures with methylene blue is known as basophilic staining.

(2) Eosin is known as an acid stain. Hb and other cytoplasmic cellular organelles are stained by eosin. Cell structures that take up acid stains are known as acidophilic or eosinophilic.

b. Wright's stain is most commonly used for routine blood film staining. It contains both basic dyes and acid stains.

(1) A good staining reaction is pH dependent (i.e., 6.4–6.7). A properly stained blood smear should demonstrate the following characteristics:
(a) RBCs should be pink or a light red.
(b) Color should be uniform.
(c) Nuclei of WBCs should stain blue to dark purple.
(d) Platelets should be clearly visible.

(2) Excessive basophilia results from over staining with the methylene blue portion of Wright's stain. Excessive acidophilia results from over staining with the eosin component of Wright's stain. RBCs will stain bright red or orange, and the WBC nuclear material will appear pale blue. Acidophilia can result from the following errors in technique:
(a) Insufficient staining time
(b) Prolonged washing time
(c) Excessive acidity of the stain or buffer

B. Phagocyte function and kinetics

1. Leukocyte maturation (Tables 4–7 and 4–8). The morphology of myeloid (i.e., granulocyte) maturation is important to know for differentiating and visually identifying cells of each maturation stage.

a. Myeloblasts are the earliest recognizable stage of myeloid maturation.

b. Promyelocytes are the second stage following one or more mitotic divisions of myeloblasts.

c. The myelocyte stage of maturation begins with the appearance of a new type of granulocyte-specific granule. These granules (secondary granules) give the mature granulocyte its characteristic appearance and functional destiny. Therefore, the maturation of the myelocyte, which is based on granule type, gives rise to neutrophils, eosinophils, or basophils.

(1) Metamyelocytes emerge after several mitotic divisions of the myelocytes.
(2) The band neutrophil stage is reached after the round metamyelocyte nucleus flattens.
(3) The final stage of a segmented neutrophil reaches full maturity once the band nucleus pinches off into segments.

2. Function of granulocytes and monocytes

a. Phagocytic function involves the following general steps:
(1) Increase in cell numbers at the infected site
(2) Cell attachment to the foreign or dead material
(3) Engulfment of foreign or dead material
(4) Dissolving of foreign or dead material
(5) Disposition of catabolic components

b. Granulocytes are the first line of defense against microbial organisms.

(1) Neutrophil migration is directed by infections or inflamed tissue.
(a) Tissue injury results in the release of vasoactive and chemotactic factors.
   (i) Vasoactive factors, such as prostaglandins and leukotrienes, increase capillary permeability and induce local migration.
### Table 4–7 Morphology of Granulocyte Maturation

<table>
<thead>
<tr>
<th>Stage</th>
<th>Cell Size</th>
<th>N–C Ratio</th>
<th>Shape</th>
<th>Chromatin</th>
<th>Nucleoli</th>
<th>Staining</th>
<th>Granules</th>
<th>Normal Range in Peripheral Blood</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myeloblast</td>
<td>10–18 μm</td>
<td>4:1</td>
<td>Round and centralized</td>
<td>Smooth</td>
<td>2–5 obvious</td>
<td>Deeply basophilic</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Promyelocyte</td>
<td>12–20 μm</td>
<td>2:1</td>
<td>Variable shape; centralized</td>
<td>Begins to condense</td>
<td>Still visible</td>
<td>Basophilic</td>
<td>Many azurophilic</td>
<td>None</td>
</tr>
<tr>
<td>Myelocyte</td>
<td>12–18 μm</td>
<td>1:1</td>
<td>Round or elongated, often eccentric</td>
<td>Slightly condensed</td>
<td>Faint</td>
<td>Mixture of basophilic and azurophilic staining</td>
<td>First appearance of secondary granules, could also be eosinophilic or basophilic granules</td>
<td>None</td>
</tr>
<tr>
<td>Metamyelocyte</td>
<td>12–15 μm</td>
<td>1:1</td>
<td>Indented, kidney-shaped</td>
<td>Slightly condensed</td>
<td>None</td>
<td>Beige or salmon staining</td>
<td>Many</td>
<td>None</td>
</tr>
<tr>
<td>Neutrophil band</td>
<td>12–13 μm</td>
<td>1:1–1:2</td>
<td>Uniform or elongated, U-shaped</td>
<td>Condensed</td>
<td>None</td>
<td>Beige or salmon staining</td>
<td>Faint</td>
<td>0%–6% or 0–700/mm³</td>
</tr>
<tr>
<td>Segmented neutrophil</td>
<td>10–14 μm</td>
<td>1:2</td>
<td>2–4 lobes</td>
<td>Highly condensed</td>
<td>None</td>
<td>Beige or salmon staining</td>
<td>Faint</td>
<td>50%–75% or 2700–7000/mm³</td>
</tr>
<tr>
<td>Eosinophil</td>
<td>12–15 μm</td>
<td>1:2</td>
<td>2–3 lobes</td>
<td>Condensed</td>
<td>None</td>
<td>Beige or salmon staining</td>
<td>Many, large, round, orange, purple granules</td>
<td>0%–4% or 0–400/mm³</td>
</tr>
<tr>
<td>Basophil</td>
<td>12–15 μm</td>
<td>1:2</td>
<td>2–3 lobes</td>
<td>Condensed</td>
<td>None</td>
<td>Beige or salmon staining</td>
<td>Many, large, round, purple granules</td>
<td>0%–2% or 0–200/mm³</td>
</tr>
</tbody>
</table>

(ii) Chemotactic factors direct the migration of neutrophils to a localized area of inflammation. Well-known chemotactic factors are activated complement C3b and C5a, lymphocyte secretions, and bacterial products, such as endotoxins or bradykinin.

(b) Neutrophils have receptors for the crystallizable fragment (Fc) portion of IgG and C3, which bind to the coated microorganism and phagocytize.

(c) Degranulation. Engulfed material is covered by internalized surface membrane, which forms a phagosome. Degranulation is the process by which

### Table 4–8 Morphology of Monocyte Maturation

<table>
<thead>
<tr>
<th>Stage</th>
<th>Cell Size</th>
<th>N–C Ratio</th>
<th>Shape</th>
<th>Chromatin</th>
<th>Nucleoli</th>
<th>Staining</th>
<th>Granules</th>
<th>Normal Range in Peripheral Blood</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monoblast</td>
<td>12–20 μm</td>
<td>4:1</td>
<td>Round with folding and clumping</td>
<td>Smooth</td>
<td>1–2 obvious</td>
<td>Deeply basophilic</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Promonocyte</td>
<td>15–20 μm</td>
<td>3:1</td>
<td>Oval, indented, or folded</td>
<td>Delicate</td>
<td>Faint</td>
<td>Basophilic with a frosted appearance</td>
<td>Few</td>
<td>None</td>
</tr>
<tr>
<td>Monocyte</td>
<td>14–20 μm</td>
<td>2:1–1:1</td>
<td>Variable</td>
<td>Slightly condensed</td>
<td>Obese</td>
<td>Blue-gray with a frosted appearance; often vacuolated</td>
<td>Few</td>
<td>3%–7% or 120–800/mm³</td>
</tr>
</tbody>
</table>

---

- **Normal**: Normal range is given for neutrophils and eosinophils. Basophils and monocytes have different normal ranges.
- **Granules**: Granules are typically present in neutrophils, eosinophils, basophils, and monocytes. The number and type of granules vary across different cell types.
- **Chromatin**: Chromatin condensation increases as cells mature, with myeloblasts having the least condensed chromatin and neutrophils having the most condensed. Eosinophils have a unique staining pattern with large, round, orange granules.
- **Shape**: The shape of granulocytes changes from round in myeloblasts to more complex shapes in later stages. Myelocytes and metamyelocytes can have indented, kidney-shaped nuclei.
- **Staining**: Staining characteristics vary across different stages, with basophilic and azurophilic stains being prominent in mature granulocytes.
activated lysosomal granules become attached to the phagosome and empty their hydrolytic enzymes into the phagosome, killing and dissolving their contents.

(d) **Bacterial killing** occurs in the phagosome by processes that are either oxygen-dependent or oxygen-independent.

(i) **Oxygen-dependent** bacterial killing in the phagosome results from the reduction of oxygen to a superoxide radical (O) in the presence of an oxidase enzyme.

(ii) Oxygen radicals bind with hydrogen to yield hydrogen peroxide (i.e., H$_2$O$_2$).

(iii) Hydrogen peroxide, with the help of chloride and iodine ions and the enzyme myeloperoxidase, dissolve the bacterial membrane.

The whole process is known as peroxidation (Figure 4–6).

(iv) Oxygen-independent killing is accomplished by hydrogen ions, lysozymes, and bactericidal proteins.

(v) **Chronic granulomatous disease (CGD)** is a fatal disorder seen in male children that results from a defect in the oxygen-dependent peroxidation pathway. The nitroblue tetrazolium (NBT) test can be used to test for leukocyte bactericidal effectiveness and to diagnose CGD.

(2) **Eosinophils** increase in circulating numbers in response to an immunologic stimuli mediated by T lymphocytes. Eosinophils decrease in number in the blood and migrate into tissue when the level of adrenocorticotropic hormone (ACTH) increases.

(a) Eosinophils phagocytize foreign particles and antigen-antibody complexes, but this is not their main function.

(b) Eosinophils may **modulate reactions** that occur when tissue mast cells and basophils degranulate.

(c) Eosinophils provide some defense against helminthic parasites.

(d) Eosinophils have a role in allergic reactions by lessening hypersensitivity reactions through the release of an **amine oxidase**, which neutralizes histamine.

(3) **Basophils** and mast cells (i.e., tissue-based basophils) are full of granules that contain histamine, heparin, and peroxidase.

(a) These cells synthesize and store histamine and eosinophil chemotactic factor of anaphylaxis (ECF-A).

(b) Basophils synthesize and release (when stimulated) substances known as slow-reacting substance of anaphylaxis (SRS-A) and platelet activating factor (PAF).

(c) Basophils are involved in hypersensitivity reactions, such as allergic asthma and delayed-onset allergy reactions.

(d) Immunoglobulin E (IgE) binds to basophil or mast cell membranes.

(i) When a specific antigen reacts with the membrane-bound IgE, degranulation occurs with the release of mediators of hypersensitivity (e.g., histamine, SRS-A, PAF, heparin, ECF-A).
(ii) ECF-A leads to an accumulation of eosinophils at the site of inflammation, which acts to counteract and balance the mediators of inflammation.

(e) Basophils are also involved in delayed hypersensitivity reactions, such as contact allergies.

c. Monocytes and macrophages

(1) The monocyte migrates into the tissue, where it is transformed into a macrophage (i.e., histiocyte).

(2) Some macrophages are motile and respond to chemotactic factors and factors from activated lymphocytes. Other macrophages are tissue-based, such as Kupffer’s cells or alveolar macrophages.

(3) Monocytes become immobilized by migration inhibiting factor (MIF), which is released from activated lymphocytes.

(4) Monocytes and macrophages become activated to phagocytize by complement, prostaglandins, or previously phagocytized material.

(5) Functions. Monocytes and macrophages are highly versatile cells that perform many vital functions, including:

(a) A defense against microorganisms such as mycobacterium, fungi, bacteria, protozoa, and viruses

(b) A role in the antigen-induced blast transformation of lymphocytes

(i) Monocytes secrete IL-1, which activates helper T cells.

(ii) Monocytes physically present and deliver the antigen to the specific membrane surface receptor sites.

(c) A major role in the daily destruction of aged blood cells, denatured plasma proteins, and lipids

(d) Heme oxidase activity, which enables some tissue macrophages to break down RBC Hb and recycle it [see Section IIIC3a(3)].

3. Granulopoiesis is controlled by glycoproteins isolated from lymphocytes and monocytes. These glycoproteins are known as interleukins (IL) and monokines, respectively. Because most research in this area was done on bone marrow cultures, these growth stimulators are also known as colony stimulating factors (CSF).

a. Multi-CSF (i.e., IL-3) production is stimulated by endotoxin released from infection.

(1) Source. IL-3 is secreted by marrow fibroblasts, T lymphocytes, macrophages, and monocytes.

(2) Function. IL-3 stimulates regeneration, maturation, and differentiation of multipotential and unipotential stem cells.

b. Granulocyte/monocyte colony stimulating factor (GM-CSF) is important for myeloid maturation in the marrow.

(1) Source. GM-CSF is secreted by T lymphocytes, marrow fibroblasts, marrow endothelial cells, and monocytes.

(2) Function. GM-CSF stimulates neutrophil, eosinophil, and monocyte growth.

c. Granulocyte colony stimulating factor (G-CSF) is a more specific granulocyte growth factor.

(1) Source. G-CSF is secreted by monocytes, marrow fibroblasts, and endothelial cells.

(2) Function. G-CSF stimulates the growth of neutrophils and enhances the functional response of neutrophils.

d. Monocyte/macrophage colony stimulating factor (M-CSF), also known as CSF-1, is the primary monocytic growth factor.

(1) Source. M-CSF is secreted by mature monocytes, marrow fibroblasts, and marrow endothelial cells.

(2) Function. M-CSF stimulates macrophages and the release of G-CSF from monocytes. This monocytic growth factor also stimulates the release of tumor necrosis factor, interferon, and IL-1 from macrophages.
C. Quantitative and qualitative leukocyte abnormalities (Figure 4–7)

1. Nonpathologic factors that affect WBC counts
   a. The age of the patient must be taken into account. At birth, the normal average WBC count is 18,000/mm$^3$.
   b. The patient’s basal condition must be taken into account.
      1. The WBC count is lowest at complete physical and mental relaxation.
      2. Exercise increases the WBC count.
   c. Physiologic conditions other than disease can affect the WBC count. Convulsions, electric shock, and pregnancy can lower the WBC count.

2. Quantitative abnormalities of leukocytes
   a. Granulocytopenia occurs with a reduction of the absolute count to lower than 3000/mm$^3$. Neutropenia results from the following physiologic mechanisms:
      1. Decreased flow of neutrophils from marrow caused by decreased or ineffective production (Figure 4–7).
      2. Increased removal of neutrophils from the blood (i.e., decreased survival; Figure 4–7).
      3. Altered distribution between the circulating leukocyte pool (CLP) and the marginal leukocyte pool (MLP).
   b. Eosinopenia is hard to detect unless a manual count is performed. Eosinopenia can result from the following conditions:
      1. Acute stress
      2. ACTH and epinephrine secretion (e.g., Cushing’s syndrome)
      3. Acute inflammatory or infectious states (i.e., shift of WBCs into the MLP and migration into inflamed tissue).
Granulocytosis refers to an absolute increase in the concentration of circulating leukocytes higher than normal for a certain age (i.e., >11,000/mm³ in adults; Figure 4–7).

(1) Physiologic neutrophilia
(a) Granulocytosis results from the following physiologic mechanisms:
(i) Increased rate of inflow of cells into the circulation from the marrow
(ii) Shift in cells from the MLP into the CLP
(iii) A decrease in the rate of outflow of cells from the blood
(b) A leukemoid reaction is a nonleukemic leukocytosis with a WBC count >30,000/mm³.
(i) This condition is characterized by the lack of myeloblasts in the peripheral blood.
(ii) A maturation shift to the left occurs with increased band forms and few metamyelocytes or myelocytes in the peripheral blood.
(iii) The LAP level will be increased, which is a distinguishing factor as compared with a leukemic granulocytosis [see Section V D 5 e (1) (c)].
(iv) Leukemoid reactions are seen most commonly in children who have infections (e.g., pneumonia, meningitis, tuberculosis) and also in cases of severe hemolysis or metastatic carcinoma.
(c) Physiologic leukocytosis is produced by factors that do not involve tissue damage:
(i) Excessive exercise, hypoxia, or stress can cause a shift from the MLP into the CLP, which appears as an increased peripheral WBC count.
(ii) Increased outflow of cells from the marrow into the blood increases both the CLP and the MLP.

(2) Pathologic neutrophilia results from disease, and is usually in response to tissue damage from a chronic infection.
(a) Three physiologic phases of shifting WBC counts can be observed in acute and chronic infections.
(i) Phase I in early infection results in the acute migration of cells from the MLP into tissue releasing chemotactic factor. Because the CLP and the MLP are in equilibrium, a transient neutropenia results. Myeloid growth factors are also released to stimulate the marrow to increase production.
(ii) Phase II is related to established chronic and severe infections when the marrow supply slowly rises to the increased demand and increases the flow of cells into the circulation. This results in a neutrophilia, a shift to the left, and a decrease in eosinophils.
(iii) Phase III is related either to recovery from the infection or to marrow exhaustion when the infection begins to subside, and the fever breaks. The total number of granulocytes decreases, and the total number of monocytes increases (i.e., secondary line of defense). If recovery does not occur, the marrow becomes depleted, and production cannot keep up with granulocyte loss. A granulocytopenia results.
(b) Miscellaneous disorders and conditions associated with neutrophilia include the following:
(i) Toxic factors that produce neutrophilia can be classified as either metabolic diseases (e.g., uremia, gout, diabetic acidosis) or as drugs and chemicals (e.g., lead, mercury, digitalis, epinephrine, ACTH).
(ii) Tissue destruction or necrosis involving a large amount of tissue can cause neutrophilia (e.g., myocardial infarction, burns, surgery, crush injuries, fractures).
(iii) Hemorrhage, if significant, can result in neutrophilia.
(iv) Acute massive hemolysis can increase the CLP.
(v) Hematologic disorders such as myeloproliferative disorders are associated with an absolute neutrophilia.
(3) Eosinophilia is clinically defined as an absolute eosinophil count >400/mm$^3$ in the peripheral blood. Disorders and conditions that demonstrate eosinophilia include the following:

(a) Allergic diseases
(b) Skin disorders
(c) Parasitic infections (e.g., Trichinosis)
(d) Infectious diseases (e.g., scarlet fever with skin rash)
(e) Pulmonary eosinophilias (e.g., Löffler’s syndrome)
(f) Blood diseases such as chronic myelogenous leukemia (CML) and other myeloproliferative disorders
(g) Splenectomy
(h) Various drugs (e.g., pilocarpine, digitalis)

(4) Basophilia is defined as an absolute basophil count >40/mm$^3$ in the peripheral blood. Basophilias have causes similar to eosinophilias.

(5) Monocytosis is defined as an absolute monocyte count >800/mm$^3$. Monocytosis is commonly observed during the recovery phase of infections and in various myeloproliferative diseases.

3. Persons who have qualitative leukocyte abnormalities demonstrate a decreased resistance to infection, despite normal leukocyte counts.

a. Lazy leukocyte syndrome is a rare inherited condition seen in children. Granulocytes do not respond to chemotactic factors; therefore, they fail to accumulate at the inflamed tissue.

b. CGD is a qualitative disorder in which the granulocytes are capable of phagocytosis and degranulation [IV B 2 b (1) (c)], but are incapable of the subsequent bacterial killing process.
   (1) Lysozymes have lost their bactericidal properties due to a decrease in the production of H$_2$O$_2$.
   (2) Granulocytes in this disorder, when stained with NBT, demonstrate a weak staining of <10% of the blood granulocytes.
   (3) The disorder is clinically characterized by frequent infections, enlarged spleen and liver, lymphadenitis, and granulomas (i.e., aggregates of mononuclear cells) in many organs.

c. Congenital and acquired qualitative disorders of neutrophils can affect either the cytoplasm or the nucleus.

   (1) Qualitative disorders affecting the neutrophil cytoplasm include the following:
      (a) Toxic granulation appears as tiny, dark blue-to-purple cytoplasmic granules in the metamyelocyte, band, or segmented neutrophil stages.
         (i) These granules are peroxidase positive.
         (ii) They are most commonly found in severe infections or other toxic conditions.
         (iii) Toxic granules are actually azurophil granules (i.e., secondary granules) that have retained their basophilic staining qualities because of lack of maturation or skipped maturation divisions during accelerated granulopoiesis.
         (iv) Cytoplasmic vacuoles are also signs of toxic change.
      (b) Döhle’s inclusion bodies are small, oval inclusions in the peripheral cytoplasm of neutrophils that stain pale blue with Wright’s stain.
         (i) Döhle’s bodies are remnants of RNA and free ribosomes or rough endoplasmic reticulum.
         (ii) These cytoplasmic inclusion stain positive with the periodic acid-Schiff (PAS) cytochemical stain.
         (iii) The appearance of Döhle’s bodies is often associated with scarlet fever, and these inclusion bodies are occasionally seen in patients who have burns; infectious diseases (i.e., often with toxic granulation); aplastic anemia; and following exposure to toxic chemicals.
(c) May-Hegglin anomaly is a rare, autosomal dominant, qualitative leukocyte abnormality.  
(i) The granulocytes of patients who have this disorder demonstrate pale blue inclusions that are larger and more prominent than the Döhle’s bodies found with infections.  
(ii) Compared with Döhle’s bodies occurring secondary to chronic infection, the cytoplasmic inclusions in May-Hegglin anomaly are PAS negative.  
(iii) Cytoplasmic inclusions result from structural RNA alterations.  
(iv) Inclusions have been found in eosinophils, basophils, and monocytes, as well as neutrophils.  
(v) Patients are seen to have a mild thrombocytopenia, giant platelets, and a slight bleeding tendency.  
  
(d) Alder-Reilly anomaly is a genetic abnormality that results in dense azurophilic granulation in all types of leukocytes.  
(i) Cytoplasmic granulation is not transient or related to an infectious disease, as it is with toxic granulation.  
(ii) Granulation results from an abnormal deposition and storage of mucopolysaccharides.  
  
(e) Chédiak-Higashi anomaly is an autosomal recessive disorder that results in qualitative abnormalities in all types of leukocytes.  
(i) Large, coarse, irregular lysosome granules are found in the cytoplasm of granulocytes and monocytes.  
(ii) Patients also demonstrate abnormal pigmentation, neuropathies, photophobia, and recurrent infections.  
(iii) Leukocytes of both the blood and bone marrow are affected.  
  
(f) Jordan’s anomaly is a genetic qualitative disorder in which abundant sudanophilic inclusions (i.e., lipid) are found in the cytoplasm of granulocytes, lymphocytes, and plasma cells in the blood and marrow.  
  
(2) Qualitative disorders affecting the neutrophil nucleus include the following:  
(a) Pelger-Huet anomaly is an autosomal dominant condition in which there is a failure of normal segmentation of neutrophil nuclei.  
(i) Most nuclei are band-shaped, have two segments, or are peanut-shaped.  
(ii) The nuclear chromatin of these cells is coarse and clumped beyond the degree of clumping found in normal immature band forms.  
(b) Hereditary hypersegmentation of neutrophils is a disorder in which the majority of the patient’s neutrophils have four or more lobes. This disorder must be differentiated from the hypersegmentation of megaloblastic anemia.  
  
(d) Monocyte-macrophage qualitative abnormalities are rare, autosomal recessive lipid-storage diseases.  
(1) Common characteristics  
(a) There is a deficiency in one of the catabolic enzymes involved in the breakdown of sphingolipids.  
(b) Because macrophages play an important role in the catabolism of lipid-rich membranes, they can accumulate undegraded lipid products in their cytoplasm. This leads to the production of lipid-heavy and blocked foamy macrophages, which stimulate further macrophage production and result in an expansion of the monocyte-macrophage system.  
(2) Gaucher’s disease is a genetic disorder that results in the accumulation of glycosphingolipids in the cytoplasm of monocytes and macrophages.  
(a) There is a deficiency of β-glucosidase, which is the enzyme that splits glucose from sphingolipids.  
(b) Macrophages and monocytes in this disease are PAS positive because of accumulated cytoplasmic carbohydrate (i.e., monocytes are normally weakly positive to negative for PAS).
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(c) Macrophage cytoplasm has a pale onion-skin appearance (i.e., Gaucher’s cells).

(3) Niemann-Pick disease is a similar disorder in which there is a deficiency in the enzyme that cleaves phosphoryl choline from sphingolipids. Macrophages and monocytes have a “foam-cell” appearance but are PAS negative.

D. Leukemias and cytochemical stains

1. Common characteristics of leukemias
   a. Incidence. Leukemia is a relatively common disorder with approximately seven new cases per 100,000 population reported annually in the United States.
   b. Definition. Leukemia can be defined as a generalized abnormal neoplastic proliferation or accumulation of hemopoietic cells in the bone marrow, organs, and the peripheral blood.
   c. Clinical classifications
      (1) Acute leukemias are refractory to remission, usually fatal within 3 months, and demonstrate a bone marrow packed with primitive cells (i.e., blasts) of the cell type involved with little differentiation.
      (2) Subacute leukemias have a longer patient survival of 3 to 12 months, and usually have a clinical picture of an acute leukemia.
      (3) Chronic leukemias are defined as having a survival of > 1 year if no remission occurs. Blasts are elevated in marrow and blood, but are usually < 5%. Maturation within a cell line still occurs.
   d. Cytologic classifications are myeloid or lymphoid.
   e. Common laboratory results include the following:
      (1) Anemia (i.e., present in more than 90% of leukemia patients)
      (2) Leukocytosis
         (a) Almost 30% of leukemic patients demonstrate normal or decreased WBC counts (i.e., aleukemic leukemia).
         (b) Approximately 20% of leukemic patients have WBC counts higher than 100,000/mm³.
         (c) The remainder of leukemic patients have elevated counts lower than 100,000/mm³.
      (3) Thrombocytopenia (present in 80% of leukemic patients)
      (4) Basophilia in the peripheral blood and bone marrow
      (5) Immature precursor or blast cells in the peripheral blood and marrow
      (6) NRBCs in the peripheral blood
      (7) Decrease in the LAP level
      (8) Nonhematologic clues such as an elevation in serum uric acid, lactate dehydrogenase, and vitamin B₁₂ levels or B₁₂-binding capacity (i.e., due to increased amounts of transcobalamin I produced by neutrophils)

2. Cytochemical stains are useful diagnostic tools (Table 4–9). The cytochemical stains help to differentiate the leukemic blasts of acute myelogenous leukemia (AML) from leukemias of lymphoid origin. It is difficult to distinguish between leukemias with Wright’s stain.
   a. The general principle of most cytochemical stains is to incubate cells on a blood smear with a substrate that reacts with an intracellular marker (e.g., lipid, lysozyme, glycogen, enzyme). In enzymatic procedures, the reaction product is coupled with a diazonium salt (i.e., dye) to produce a visible reaction product.
   b. Sudan black B and peroxidase (i.e., myeloperoxidase) cytochemical stains share a common staining pattern.
      (1) Sudan black B stains phospholipids and sterols (i.e., lipids) in cytoplasmic lysosome and mitochondrial membranes
         (a) Azurophilic and specific neutrophil granules are stained.
         (b) Cytoplasmic granules stain faintly in myeloblast cells and strongly in mature neutrophils. As the cell matures from the promyelocyte stage to the mature segmented neutrophil, each stage is generally more positive.
   e. Eosinophilic granules stain positive.
### Table 4–9: Cytochemical Staining Reactions in Leukemias and Individual Cells

<table>
<thead>
<tr>
<th>Cells</th>
<th>Sudan B Black</th>
<th>Chloracetate Esterase</th>
<th>α-Naphthyl Esterase</th>
<th>Acid Phosphatase</th>
<th>Acute Leukemias</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutrophil</td>
<td>P</td>
<td>P</td>
<td>N</td>
<td>P</td>
<td>WP</td>
</tr>
<tr>
<td>Precursor</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>WP</td>
</tr>
<tr>
<td>Myeloid</td>
<td>P</td>
<td>P</td>
<td>N</td>
<td>WP</td>
<td>AML</td>
</tr>
<tr>
<td>Lymphocyte</td>
<td>N</td>
<td>N</td>
<td>WP</td>
<td>P</td>
<td>AML-M4</td>
</tr>
<tr>
<td>Monoblast</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>WP</td>
<td>F</td>
</tr>
<tr>
<td>ALL</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normoblast</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>WP</td>
<td>AML-M6</td>
</tr>
</tbody>
</table>

- ALL = acute lymphocytic leukemia; AML = acute myelogenous leukemia; AML-M4 = acute monoblastic leukemia; AML-M6 = erythroleukemia. + = positive in erythroleukemia. − = focal positivity. N = negative staining; P = strongly positive staining; WP = weakly positive staining; (−) = leukemia blasts stain negative; (+) = leukemia blasts stain weakly positive; 1+ = a few leukemia blasts stain positive; 2+ = leukemia blasts stain moderately positive; 3+ = leukemia blasts stain strongly positive.

(d) Monocytes are generally unstained or weakly positive with a few scattered positively stained cytoplasmic granules.

(e) Lymphoblasts and lymphocytes are negative.

(f) Leukemic myeloblasts demonstrate stronger positivity than normal blasts.

(g) Auer bodies in leukemic myelogenous cells stain positive.

(2) Peroxidase stains only azurophilic granules.

(a) The stain reacts with the lysosomal enzyme in azurophilic granules to yield a black or brown reaction product.

(b) Reactions with myeloperoxidase stain are generally the same as that for Sudan black B.

(c) Esterase cytochemical stains include either specific or nonspecific esterase procedures.

(1) AS-D chloroacetate esterase stain (specific esterase) is more useful in separating monocyte precursors from granulocyte precursors than are the Sudan black B or peroxidase cytochemical stains.

(a) Neutrophils and neutrophil precursors stain the most strongly positive of all cell lines.

(b) Generally, monocytes, lymphocytes, and their precursors stain negative.

(2) α-Naphthyl acetate esterase stain (nonspecific esterase) is also useful to differentiate neutrophil precursors from monocytes and their precursors in acute leukemias.

(a) Monocytes stain strongly positive at all stages of maturation. The addition of sodium fluoride to the incubation solution inhibits the staining reaction in monocytes but not in granulocytes.

(b) Megakaryocytes and macrophages stain positive.

(c) Granulocytes at all stages of maturation stain negative or only weakly positive.

(d) Basophils, plasma cells, and T lymphocytes stain positive.

(d) PAS is often useful for identifying some lymphocytic leukemias.

(1) Periodic acid (HIO4) is an oxidizing agent that converts hydroxy-groups on adjacent carbon atoms to aldehydes (e.g., carbohydrates).

(a) The resulting reaction product is red-colored at the site of hydroxy-group conversion.

(b) A positive reaction is seen in the presence of polysaccharides, mucopolysaccharides, glycoproteins, and other carbohydrates.

(c) A positive PAS reaction usually indicates the presence of stored carbohydrates in the cytoplasm of a cell.

(2) Neutrophils are positive at most stages of development, but most strongly in the mature stage. Myeloblasts are usually weakly positive or negative.
Eosinophils are positive at all stages of development.

Monocytes are weakly positive in the form of fine granules.

Lymphocytes may contain a few positive granules, but in lymphocytic leukemias the malignant lymphocytes may have an increased number of PAS-positive granules in a focal or block-like positivity.

NRBCs are negative, but stain positive in the abnormal erythroid precursors of erythroleukemia.

Megakaryocytes stain positive.

e. Acid phosphatase cytochemical staining is useful for confirming a diagnosis of hairy-cell leukemia and for differentiating a T-lymphocytic leukemia from a B-lymphocytic leukemia.

(1) Acid phosphatase-containing cytoplasmic granules stain red.

(2) Monocytes stain strongly positive.

(3) Neutrophils and precursors stain positive but less intensely than do monocytes.

(4) T lymphocytes stain positive, but B lymphocytes stain negative.

(5) A variation of this staining procedure calls for the addition of L-tartaric acid into the staining reagent. This variation is known as the tartaric acid resistant acid phosphatase stain, or the TRAP stain.

(a) L-tartaric acid used in the staining solution inhibits the isoenzymes of acid phosphatase, and therefore results in a negative reaction in most cell types that normally stain positive.

(b) However, the acid phosphatase isoenzymes in the malignant lymphocytes of hairy-cell leukemia are not inhibited by the addition of L-tartaric acid and will still stain positive.

f. LAP staining is useful for distinguishing a leukemic neutrophilia from a non-leukemic neutrophilia seen with chronic inflammation and infection.

(1) This enzyme is found in neutrophils from the myelocyte stage to the mature segmented neutrophil stage.

(2) The enzyme is detected in its reaction with a naphthyl phosphate in the presence of a diazonium salt (e.g., fast blue or fast violet) at an alkaline pH of 9.5.

(3) After staining, a Kaplow count is performed. A total of 100 mature neutrophils are scored from 0 (negative) to 4 (strongly positive), based upon the intensity of the staining reaction. An example of how a count is scored is demonstrated in Box 4–4.

(4) The scores will range from a score of 0 to 400. Reference values are usually in the range of 20 to 200, although each hospital usually establishes its own normal range.

(a) Increased LAP activity occurs in the following diseases or conditions:

(i) Infections

(ii) Polycythemia vera

(iii) Hodgkin’s disease

(iv) Myelofibrosis with myeloid metaplasia

(v) Pregnancy (i.e., last trimester)

<table>
<thead>
<tr>
<th>Staining intensity</th>
<th>Number of cells</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1+</td>
<td>35</td>
<td>35</td>
</tr>
<tr>
<td>2+</td>
<td>15</td>
<td>30</td>
</tr>
<tr>
<td>3+</td>
<td>3</td>
<td>9</td>
</tr>
<tr>
<td>4+</td>
<td>2</td>
<td>8</td>
</tr>
</tbody>
</table>

Kaplow count: 82
E. Chronic and acute myeloproliferative disorders (Figure 4–8)

1. Characteristics. Myeloproliferative disorders are a group of closely related diseases characterized by the spontaneous proliferation of erythroid, granulocyte, monocytic, or megakaryocyte precursors in the marrow. Some shared general characteristics include the following:
   a. The spleen, liver, and lymph nodes may be involved.
   b. All cell lines or only a single cell line may be involved.
   c. Myeloproliferative disorders are clonal in origin, having arisen from a single pluripotential hematopoietic stem cell.
   d. Cytogenetic abnormalities are common to most myeloproliferative disorders (Table 4–10).

2. Chronic myeloproliferative disorders include a group of leukemias that share a common stem cell lesion and are slow in their clinical course.
   a. CML occurs mainly in middle-aged adults with a slow and unrevealing onset of symptoms.
      (1) Clinical symptoms, which are mainly caused by the body’s increased load of myeloid cells and nutritional demands, include the following:
         (a) Anemia
         (b) Weight loss
         (c) Lack of energy
         (d) Spleen enlargement causing abdominal discomfort
         (e) Fever
         (f) Excessive bleeding or bruising (i.e., due to decreased platelet production)
Table 4–10 Common Cytogenetic Abnormalities of Chronic and Acute Myeloproliferative Disorders as Compared to Their Common Monoclonal Immunofluorescent CD Markers

<table>
<thead>
<tr>
<th>Myeloproliferative Disorder</th>
<th>Cytogenetic Abnormality</th>
<th>CD Marker</th>
</tr>
</thead>
<tbody>
<tr>
<td>CML</td>
<td>Ph1, t(9:22)</td>
<td>CD11b, CD33</td>
</tr>
<tr>
<td>AML-M2</td>
<td>t(8:21)</td>
<td>CD11b, CD33</td>
</tr>
<tr>
<td>AML-M3</td>
<td>t(15:17)</td>
<td>CD11b, CD33</td>
</tr>
<tr>
<td>AML-M4</td>
<td>t(16q)</td>
<td>CD11b, CD14, CD33</td>
</tr>
<tr>
<td>AML-M5</td>
<td>t(9:11)</td>
<td>CD11b, CD14, CD33</td>
</tr>
<tr>
<td>MDS</td>
<td>-5, 5q-, -7, 7q-, trisomy 8, and 12 q</td>
<td>No data</td>
</tr>
</tbody>
</table>

AML = acute myelogenous leukemia (subdivided as M1-M7); AML-M4 = acute myelomonocytic leukemia; AML-M5 = acute monocytic leukemia; CD = cluster designation; CML = chronic myelogenous leukemia; MDS = myelodysplastic syndromes; Ph1 = Philadelphia chromosome.

(2) A patient who has CML typically is seen with the following blood and marrow laboratory profile:
(a) A WBC count usually >50,000/mm³ and possibly as high as 300,000/mm³.
   (i) CML has a characteristic differential count showing complete maturation of granulocyte cells from myeloblasts to segmented neutrophils and a bimodal distribution with myelocytes and segmented neutrophils both exceeding other types in absolute numbers. Myeloblasts are usually <10% in the blood and marrow.
   (ii) Basophilia is almost always present.
   (iii) An absolute eosinophilia and monocytosis are also typical.
   (iv) Normocytic/normochromic anemia occurs in most cases due to decreased RBC production.
   (v) Thrombocytosis is seen in the early phases of the disease, and thrombocytopenia occurs in later phases.
(b) Bone marrow is hypercellular because of granulocyte proliferation with all stages of maturation represented.
   (i) Myeloid-erythroid ratios of 15:1 to 50:1 are common.
   (ii) Eosinophil and basophil precursors are often increased.
   (iii) Blasts are increased, but usually <10%.
(c) LAP is reduced or absent in more than 90% of CML patients. In one third of CML patients who are in remission, the LAP level returns to normal.
(d) Cytogenetic abnormalities found in CML are highly diagnostic.
   (i) Direct bone marrow preparations show that 90% of CML patients demonstrate the “Philadelphia” (Ph1) chromosome.
   (ii) The mutation results from a translocation from the long arm of chromosome 22 to an arm of chromosome 9.
   (iii) The 10% of CML patients who are Ph1 negative have similar laboratory and clinical findings. However, this group is characterized by the following: a larger proportion of children, not as good a response to therapy, and a shorter survival.
(e) Serum levels of vitamin B12 and B12-binding proteins are increased because of the increased total granulocyte pool.
(f) Serum uric acid and muramidase are increased.
(3) CML is treated with chemotherapeutic agents such as busulfan, which is an alkylating agent, or hydroxyurea, which is a folic acid antagonist.
(a) Alkylating agents are thought to combine with guanine in DNA to inhibit rapidly growing cells.
(b) Toxic effects of chemotherapy occur, such as bone marrow depression and bleeding.
(4) Remissions may last from several months to several years. The LAP level returns to normal, and the Ph1 chromosome usually disappears.
(a) When the patient comes out of remission, the disease changes into a more aggressive and accelerated phase characterized by a rising granulocyte count and spleen enlargement.

(b) When blasts exceed 30% of cells in the marrow or blood, it is known as the blastic phase of the disease, or blast crisis.

b. Polycythemia vera (PV) is characterized by excessive proliferation of erythroid, granulocytic, and megakaryocytic elements of the marrow, and an increase in circulating RBC mass (Figure 4–1).

(1) The neoplasia is classified as an absolute primary polycythemia and differentiated from secondary polycythemia by the hyperproliferation in all cell lines.

(2) A patient who has PV typically presents with the following blood and marrow laboratory profile:

(a) The peripheral blood typically has an elevated RBC count between 6.0 and 12.0 × 10^6/mm^3.
   (i) The Hb is elevated between 18 g/dL and 24 g/dL, and the MCV, MCH, and MCHC are normal to low.
   (ii) Blood smear RBC morphology shows some macrocytes, polychromatic cells, and normoblasts.
   (iii) RBC production is increased, and RBC survival is normal, but RBC survival is reduced if the spleen is enlarged.
   (iv) Oxygen Hb saturation is normal.
   (v) Patients have a leukocytosis of 10,000 to 30,000/mm^3, and the presence of immature myeloid and erythroid cells on their peripheral blood smear.
   (vi) This disorder demonstrates an increased level of LAP.
   (vii) The serum vitamin B12 level and binding capacity are increased.
   (viii) Patients who have PV have decreased levels of serum and urine EPO. This can be a diagnostic characteristic, because all secondary polycythemias are the result of an increased level of EPO.

(b) Marrow aspirate demonstrates a hypercellular marrow with all cell lines increased in absolute amounts. Iron storage is decreased or absent.

(3) Treatment of PV is with one or a combination of the following methods:

(a) Phlebotomy
(b) Chemotherapy
(c) Radioisotope phosphorus 32

(4) A late complication of PV is acute leukemia, which is often caused by treatment with alkylating drugs and radiotherapy.

(a) In 20% to 40% of individuals who have PV, progressive anemia, splenomegaly, and further leukocytosis occurs with more circulating immature granulocytes, normoblasts, and dacryocytes apparent on blood smears.

(b) Myelofibrosis in the marrow may increase.

(c) Many cases of PV evolve into myeloid metaplasia with myeloid metaplasia (MMM).

c. MMM is characterized by a chronic, progressive pancytopenia with varying fibrosis of the marrow.

(1) Patients demonstrate a massive splenomegaly caused by extramedullary hematopoiesis (i.e., MMM).

(2) A patient with MMM typically demonstrates the following blood and bone marrow laboratory profile:

(a) The peripheral blood shows a moderate normocytic-normochromic anemia with some basophilic stippling.
   (i) RBC morphology shows moderate anisocytosis and poikilocytosis (e.g., fragmented forms; dacryocytes; elliptocytes; and NRBCs).
   (ii) Reticulocytosis is common.
The leukocyte count is normal or slightly increased, and the differential shows a few immature granulocytes.

(iv) The LAP level is increased.

(v) Platelet counts can be normal or decreased with the presence of abnormal platelets.

(vi) The serum uric acid level is increased, but the serum vitamin B₁₂ level is usually normal.

(b) Marrow aspiration is usually “dry” because of the increase in reticulin fibers and patchy fibrosis.

(3) The usual course of MMM is progressive anemia, enlargement of the spleen, and opportunistic infections.

d. Essential thrombocythemia is a clonal malignancy most closely related to PV. It is characterized by a predominance of megakaryocytic proliferation in the marrow.

(1) Clinically, patients are often seen with reoccurring, spontaneous hemorrhages (i.e., mostly GI in origin).

(2) A patient who has thrombocythemia typically demonstrates the following blood and bone marrow laboratory profile:

(a) The peripheral blood shows a marked increase in platelets (between 900,000/mm³ and 1,400,000/mm³) with abnormal giant forms and fragments of megakaryocytes.

(i) Patients have a leukocytosis.

(ii) The LAP level is normal.

(iii) Many patients demonstrate a hypochromic-microcytic anemia due to chronic blood loss.

(iv) Platelet function defects can be present, and platelets may show a decreased aggregation in response to epinephrine.

(b) A hypercellular marrow demonstrates a pancytopenia with increased megakaryocytes and increased reticulin (i.e., may result in a “dry” aspiration).

(3) Course. Thrombocythemia has a stable course for many years, but may develop into other myeloproliferative disorders.

e. Myelodysplastic syndrome (MDS) is a family of marrow disorders found mainly in persons older than 50 years. MDS is characterized by ineffective cellular production.

(1) MDS is believed to be caused by a defect in a member of the marrow stem cell pool, which results in increased proliferation and inadequate maturation or an imbalance in one or more cell lines.

(2) This group of disorders has been termed preleukemias because most patients progress to have acute leukemias.

(3) Between 40% and 90% of MDS patients have demonstrated chromosome abnormalities. Mutations have been commonly found on chromosome 5, but also have been noted on chromosomes 7, 8, 12, and 20.

(4) MDS is classified into five subtypes, as defined by the French-American-British (FAB) Cooperative Pathology Group.

(a) Refractory anemia (RA) has the following identifying characteristics:

(i) Anemia with a decreased reticulocyte count

(ii) Abnormal erythrocytes (e.g., ovalocytes)

(iii) Blasts < 1% in peripheral blood

(iv) Approximately 10% of RA patients progress to AML.

(b) Refractory anemia with ring sideroblasts (RARS) has the following identifying characteristics:

(i) Greater than 15% ring sideroblasts in the marrow (i.e., any nucleated erythroid precursor cell that contains stainable iron granules)

(ii) Ring sideroblasts have a “necklace” of iron granules around the nucleus

(iii) Approximately 10% of RARS patients progress to AML
(c) Refractory anemia with excess blasts (RAEB) has the following identifying characteristics:
   (i) A cytopenia in two of the three cell lines
   (ii) Greater than 1%, but <5% circulating blasts
   (iii) Between 5% and 20% blasts in the marrow

(d) Chronic Myelomonocytic leukemia (CMML) has the following identifying characteristics:
   (i) Chronic monocytosis >1,000/mm$^3$
   (ii) Frequent granulocytosis
   (iii) Less than 5% circulating blasts
   (iv) Greater percent of promonocytes seen in marrow

(e) RAEB in transformation (RAEBIT) has the following identifying characteristics:
   (i) Greater than 5% circulating blasts
   (ii) Between 20% and 30% blasts in the marrow
   (iii) Presence of Auer bodies in blasts
   (iv) Approximately 60% of patients with RAEBIT transform into AML

3. Acute myeloproliferative leukemia (AML) includes a group of leukemias that share a common stem cell lesion, are more refractory to treatment, and have a rapid onset in their clinical course.

a. Patients diagnosed with AML share the following common clinical symptoms and laboratory features:
   (1) AML is a disease of adulthood.
   (2) Many cases are believed to be viral-induced or can be related to exposure to radiation, chemicals, or diseases with a long preleukemic phase.
   (3) Most patients have very high WBC counts (e.g., 200,000/mm$^3$).
   (4) AML is resistant to treatment with chemotherapeutic agents.
   (5) The disease has a rapid onset with symptoms often resembling an acute infection.
   (6) Ulcerations of mucous membranes are commonly seen.
   (7) Patients have a lack of energy.
   (8) Symptoms are due to the mass of leukemic cells blocking capillaries, crowding out normal bone marrow cells, and compromising the immune system, all resulting in anemia, hemorrhage, and infections.
   (9) In 1976, the FAB group published guidelines for the classification of AMLs based on the morphology of the cells involved. This classification system has come to be known as the FAB classification of AML and represents seven subtypes (M1 to M7).

b. AML-M1 and AML-M2 are known as acute myeloblastic leukemias.
   (1) M1 is defined by a predominance of myeloblasts in the marrow with very little maturation beyond the blast stage. Cytochemical staining reactions occur, as illustrated in Table 4–9.
   (2) AML-M2 is different from M1 because of the finding of a significant number of myeloid precursor cells in the marrow beyond the blast and promyelocyte stages (i.e., maturation is involved).
      (a) More than 50% of marrow cells are myeloblasts or promyelocytes.
      (b) Patients who have M2 have a common chromosome abnormality of t(8;21).
   (3) Maturation abnormalities are often present with M1 or M2.
      (a) Hypossegmentation is seen in many of the neutrophils (i.e., pseudo-Pelger Huët).
      (b) Myeloblasts can show bilobed or reniform nuclear shapes.
      (c) Some blasts may present with azurophilic granules.
      (d) Auer bodies can be found in the cytoplasm of a few blasts.
         (i) These inclusions are linear or spindle-shaped, red-purple inclusions in the cytoplasm of blasts or promyelocytes.
(ii) They are thought to be giant lysosomes, and they stain positive for Sudan black B, peroxidase, AS-D chloroacetate esterase, and acid phosphatase.

c. **AML-M3** is known as **Acute Promyelocytic Leukemia (APL)**. Cytochemical staining reactions are summarized in Table 4–9.

1. Promyelocytes instead of myeloblasts are the major abnormal cell found in the marrow and blood.
2. These promyelocytes have an abundance of azurophilic granules.
3. Auer bodies are found in most patients who have M3.
4. Two variants of APL are found based on the staining intensity of promyelocyte granularity.
   (a) **Hypergranular promyelocytes** are the most common variety of M3.
      (i) Characteristically, promyelocytes may have bundles of cytoplasmic Auer bodies and intensely staining azurophilic granules.
      (ii) The nucleus of these promyelocytes may vary in size and shape (i.e., kidney-shaped or bilobed).
      (iii) Patients who have hypergranular M3 often have bad bleeding problems. DIC is easily initiated by procoagulant material released from granules of abnormal hypergranular promyelocytes.
      (iv) The WBC count is not usually as greatly elevated as that seen with other AMLs.
   (b) Hypogranular promyelocytes (M3V) may be easily confused with myeloblastic leukemia (i.e., M1 or M2) because promyelocytes are faintly granulated and may appear as myeloblasts.
      (i) In approximately 60% of patients who have M3V, a t(15;17) chromosome translocation is demonstrated.
      (ii) Patients who have this variant usually demonstrate very high WBC counts (up to 200,000/mm³).

d. **AML-M4**, **acute myelomonocytic leukemia (AMML)**, demonstrates a predominance of both monocyte and granulocyte precursor cells in the marrow and peripheral blood.

1. This leukemia is defined as a malignancy in which more than 20% promonocytes and monocytes are found in marrow and peripheral blood with the presence of more than 20% myeloblasts and abnormal granulocyte precursor cells.
2. Chromosome abnormalities are seen as a 16q mutation.

e. **AML-M5** is a relative pure monocytic leukemia.

1. This leukemia is defined as a malignancy in which more than 20% promonocytes and monocytes, and <20% myeloblasts and granulocytic precursors are found in the marrow or peripheral blood.
2. Two subtypes of M5 are found:
   (a) **Differentiated monoblastic leukemia (M5a)** has monocytes and promonocytes as the majority in the marrow and blood. The blood has a higher percentage of monocytes than the marrow, and the most common type of cell is the promonocyte.
   (b) Poorly differentiated (M5b) is characterized by a predominance of large blasts in the bone marrow and blood.
3. Chromosome abnormalities are seen in M5 as a t(9;11).
4. Promonocytes and leukemic monoblasts in AML-M5 demonstrate the following morphologic characteristics:
   (a) Delicate reticular chromatin
   (b) One to two nucleoli
   (c) Folded and indented nuclei
   (d) Moderate nucleus:cytoplasm (N:C) ratio (i.e., 1:1 to 2:1)
   (e) Phagocytosis of RBCs or cellular debris
   (f) Auer bodies
   (g) Abundance of basophilic-staining cytoplasm with pseudopodia and possibly rare azurophilic granules
AML-M5 may have lymph node enlargement.

Cytochemical staining reactions for M4 and M5 can be found in Table 4–9.

Patients who have monocytic leukemias characteristically have very high levels of muramidase in the serum or urine.

AML-M6 or erythroleukemia was formerly known as Di Guglielmo syndrome. This type of AML results from an abnormal proliferation of both erythroid and granulocytic precursors with a predominance of erythroid precursors in the marrow representing 50% or greater of the marrow precursor cell population. Myeloid-erythroid ratios decrease to between 1:2 and 1:4.

Erythremic myelosis is a variant of M6 that has a more rapid course and an arrest in the maturation of the erythroid precursors. Mainly, only the erythroblast stage of maturation is seen in the erythroid marrow. Often, M6 terminates in AML-M1.

Erythroid precursors are abnormal in appearance, predominate in the marrow and blood, and demonstrate the following cellular morphologic characteristics:

- Blasts irregular in outline, often with pseudopodia
- Medium N:C ratio
- Bizarre large nucleoli and nuclear shape
- Numerous multinucleated giant forms
- Cytoplasmic vacuolation

Myeloblasts are increased in the chronic form of erythroleukemia, and Auer bodies may be present. Abnormal megakaryocytes are present with giant forms and nuclear fragmentation. Atypical platelets may also be found in the blood.

The cytochemistry results of M6 show a strong positivity to PAS. Erythroid precursors are normally PAS negative.

Patients with AML-M6 have a poor prognosis.

AML-M7 or Acute Megakaryoblastic Leukemia (i.e., acute myelofibrosis) is the newest FAB classification of acute nonlymphocytic leukemias. This leukemia demonstrates a predominance of megakaryoblasts and megakaryocytes in the marrow with an increase in reticulum fibrosis. M7 is the rarest subtype.

As a group, AMLs generally have a terminal prognosis of approximately 3 to 6 months, and complete remission is achieved in 40%–60% of patients.

Rare forms of leukemia

- Acute stem cell leukemia is a leukemia in which the predominant hyperproliferating cells are blast forms that cannot be classified using cytochemical or immunologic techniques.
- Chloroma is a rare form of AML in which green-pigmented tumors of myeloblasts form in the tissues surrounding the bones.
- Myeloblastic sarcoma is a form of AML in which localized tumors of myeloblasts are found in tissues. This type differs from chloroma only in the absence of the green pigment found in the tumor.
- Eosinophilic leukemia can occur, although rarely, as a variant of AML or CML. Types. There are acute and chronic forms. Laboratory profile. Immature eosinophils infiltrate the tissues in the body. Other immature myeloid precursor cells are usually involved, and their levels are increased in the blood and marrow. Differential diagnosis. This disorder must be distinguished from hypereosinophilic syndrome seen with parasitic infection. Diagnosis. The identification of immature forms in the blood and an increase of eosinophils in the marrow of >5% help diagnose eosinophilic leukemia.
- Basophilic leukemia can be a variant of CML in the accelerated phase. This variant is associated with an increase in blood histamine levels.
V. LYMPHOCYTE PHYSIOLOGY AND DISORDERS

A. Lymphocyte development
1. Development involves three major lymphoid compartments.
   a. The pool of undifferentiated stem cells is located in the bursa-equivalent tissue of the bone marrow, where proliferation and maturation occurs.
   b. Primary lymphoid tissues are the location of antigen-independent lymphopoiesis.
      (1) T-lymphocyte development occurs in the thymus (Figure 4–9).
      (2) B-lymphocyte development occurs in the bone marrow.
   c. Peripheral or secondary lymphoid development is antigen dependent. Secondary lymphoid tissues contain mixed T-cell and B-cell populations; these tissues are found in the spleen, lymph nodes, and gut-associated lymphoid tissue.
2. B-lymphocyte development (see Chapter 6, Section II B)
3. T-lymphocyte development is centered in the thymus gland (see Chapter 6, Section II A).

B. Lymphocyte structure
1. From a morphologic basis, lymphocyte development consists of three recognized stages.
   a. Lymphoblasts are the youngest recognizable form of B lymphocytes or T lymphocytes based on staining characteristics.
      (1) Blast size is larger than a mature lymphocyte, averaging 16–24 mm.
      (2) Blasts have 1 to 2 easily recognizable large nucleoli.
      (3) Chromatin in these blasts is finely divided into a smooth grainy texture.
      (4) Lymphoblasts have the highest N:C ratio of any blast cell.
      (5) Cytoplasm is scant and highly basophilic in staining.
   b. The prolymphocyte is a middle stage in the development between the blast and mature lymphocyte.
      (1) The size of this mid-stage cell is the same or slightly larger than the blast, averaging 18 to 28 μm.
      (2) Nucleoli can still be easily recognized.
      (3) The nuclear chromatin pattern is slightly more condensed than the blast stage.
(4) Prolymphocytes have a high-to-moderate N:C ratio.
(5) The cytoplasm is more abundant than the blast but still deeply basophilic.

c. Mature lymphocytes in the peripheral blood represent non-dividing B lymphocytes or T lymphocytes between the primary and secondary development.
(1) Size. Small lymphocytes are just slightly larger than an RBC (i.e., 8–12 μm).
(2) Organelles. These cells have a sharply defined, round nucleus that contains heavy concentrations of dense chromatin appearing blocked or “smudgy.” The nucleus stains deep blue to purple and is sometimes indented at one side. The cytoplasm stains a pale blue. Normally, lymphocytes do not demonstrate specific cytoplasmic granules.
(3) In a small lymphocyte, the nucleus occupies most of the cell area, and there may be just a thin perinuclear zone of cytoplasm.
(4) Large lymphocytes may be found in the peripheral blood, especially in children. These lymphocytes demonstrate the following morphologic characteristics:
(a) 12 to 15 μm in diameter
(b) Abundant cytoplasm
(c) Nuclei less densely staining
(d) Irregular borders and shape
(e) May appear as a small monocyte
(f) Few bluish purple cytoplasmic granules
(5) Lymphocytes average 25% to 45% of all circulating WBCs with a total absolute count range of 1,000 to 4,800/mm³.
(6) T and B lymphocytes cannot be distinguished from each other morphologically.

d. Plasma cells originate from B lymphocytes. They are cells designed for the synthesis of immunoglobulin.
(1) Plasmablasts, or immunoblasts, have a large blast-like nucleus with nucleoli, but have other plasma cell characteristics.
(2) Plasma cells are identified by their eccentrically placed nucleus and the following morphologic characteristics:
(a) The nucleus is round with a small indentation on the cytoplasm side.
(b) Nuclear chromatin is distributed more regularly in a pattern resembling the spokes of a wheel.
(c) The cytoplasm, except for one small area, stains deeply bluish green.
(d) The unstained portion of cytoplasm corresponds to the indented portion of the nucleus. A large golgi apparatus exists here because of the large amount of protein synthesis.

2. Lymphocytes demonstrate a heterogeneity of functional types.

a. B lymphocytes function as precursors for plasma cells. They also synthesize and release immunoglobulin, which is easily detectable on their surface membranes.
(1) Approximately 10% to 15% of circulating blood lymphocytes are B cells.
(2) Diversity
(a) Individual B cells are limited to the one type of antibody they can synthesize and release.
(b) IgM or immunoglobulin D (IgD) are found on most circulating B cells.
(c) B lymphocytes with surface IgM or IgG are found mainly in organized secondary lymphoid tissue.
(d) Immunoglobulin A (IgA)- and IgE-bearing B cells are predominately at sites of external Ig secretion (e.g., the GI and respiratory tracts, saliva).
(3) B-lymphocyte surface receptors include the following identifying markers:
(a) Fc receptors (i.e., CD7) that recognize the Fc portion of immunoglobulins
(b) C3 receptors (i.e., CD21) for complement fixation
(c) Specific receptor for the Epstein-Barr virus antigen
(d) Mouse RBC receptor

b. T lymphocytes are responsible for reactions of cellular immunity and modulation of humoral immunity (Figure 4–9).
T cells interact with macrophages for the proper delivery of antigens to B lymphocytes.

T cell-mediated immunity is antigen directed; therefore, T cells must have surface receptors to recognize antigens.

(a) T lymphocytes display a specific receptor for sheep RBCs (i.e., CD2), forming E-rosettes.

(b) Fc receptors are present but not abundant.

d) CD1 to CD8 are also abundant.

c. Null lymphocytes are lymphocytes that cannot be classified as either T or B types on the basis of surface properties. These cells constitute approximately 10% of the lymphocyte population.

1. Null cells possibly represent undifferentiated stem cells, immature T or B cells, or those lymphocytes that have lost their recognizable surface receptors.

2. L cells are lymphocytes that do not proliferate in response to antigens but are capable of enhancing the responses of T lymphocytes in the presence of monocytes.

(a) These lymphocytes bear surface IgG and Fc receptors.

(b) L cells do not develop into antibody-producing cells.

3. Large granular lymphocytes (LGLs) mediate antibody-dependent cytotoxicity.

(a) LGLs include natural killer (NK) and killer (K) cells.

(b) NK cells mediate cytotoxic reactions without prior sensitization in defensive mechanisms against tumors and virally transformed cells.

C. Function and regulation of the immune response

1. Monocyte-macrophages secrete IL-1, which activates the helper-inducer T lymphocytes. IL-1 also has pyogenic effects on the central nervous system (CNS) in raising body temperature.

2. Helper-inducer T lymphocyte lymphokines, which are secreted in an immune reaction (see Chapter 6, Section II A), consist of the following substances:

   a. Once activated by IL-1, the helper-inducer T lymphocyte secretes IL-2, which stimulates other helper-inducer T lymphocytes to multiply.

   b. B-cell growth factor (BCGF) is released to stimulate B-cell proliferation.

   c. B-cell differentiation factor (BCDF) is released to halt replication of the immunospecific B cells and stimulates antibody production.

   d. γ interferon is released to stimulate B-cell antibody production, activate killer T lymphocytes, and localize macrophages at the site of infection.

3. B lymphocytes (see Chapter 6, Section II B) produce and secrete an antigen-specific antibody.

D. Lymphocyte pathophysiology

1. The normal circulating concentration of lymphocytes is 1,500 to 4,000/mm³.

   a. An absolute circulating lymphocyte count below 1,500/mm³ is considered clinically to be a lymphocytopenia.

   b. The reduction in circulating lymphocytes affects mostly T cells, because they represent the greatest percentage of circulating lymphocytes.

2. Nonmalignant disorders of lymphocytopenia often include a hypogammaglobulinemia (Figure 4–10). A lymphocytopenia can be found in the following disorders or conditions:

   a. A blockage of the lymphatic thoracic duct results in a lymphocytopenia.

   b. Radiation overexposure is highly toxic to lymphopoiesis and results in a reduction of lymphocytes.

   c. Acute stress results in a reduction of lymphocytes in the circulating pool.

   d. Therapy with corticoids shifts distribution of lymphocytes from the blood into the extravascular spaces. Cell lysis and an inhibition of cell proliferation are minor drug-induced mechanisms of lymphopenia.

   e. Chemotherapeutic alkylating drugs interfere with lymphocyte proliferation.

   f. Acquired immunodeficiency syndrome (AIDS) is a virally induced lymphocytopenia.
The virus that causes AIDS has been named human T-cell lymphotrophic virus-3 (HTLV-3) or human immunodeficiency virus (HIV). Patients who have AIDS have a high risk of infections because of a significant decrease in helper-inducer T lymphocytes. The AIDS virus invades the helper-inducer T cells and renders them incapable of functioning. The T lymphocyte helper-suppressor (H-S) ratio is decreased below the normal range of 0.9% to 2.9%.

Primary immunodeficiency disorders include several disorders caused by a developmental defect in either B or T lymphocytes. X-linked agammaglobulinemia (Bruton type) is a developmental defect of B cells that primarily affects male infants. B-cell zones of lymph nodes and spleen are depleted of B cells. Blood lymphocyte counts are normal, but the serum immunoglobulin concentrations are very low, and patients suffer from recurrent infections.

Hypogammaglobulinemia of infancy is a decrease in immunoglobulins caused by delayed immune development in the first years of life. Normally, following the gradual disappearance of maternal IgG, an infant’s own IgG and IgM levels increase to approximately 75% of the adult level by 1 year of age. Infants who have this disorder have a delayed onset of immunoglobulin synthesis and are subject to recurrent infections.

Thymic aplasia (Di-George’s syndrome) is a developmental defect of the thymus gland. T-cell zones of the lymph nodes are depleted.

Selective immunoglobulin deficiency is an acquired decrease in one subtype of immunoglobulin. A lack of IgA is most common. Thymic aplasia (Di-George’s syndrome) is a developmental defect of the thymus gland.
(6) Severe combined immune deficiency is a defect in the common stem cell that leads to a deficiency of both T and B lymphocytes.

2. Lymphocytosis is clinically defined as an absolute circulating increase in the lymphocyte count >4,000/mm³ in adults, 7,000/mm³ in children, and 9,000/mm³ in infants. Disorders that demonstrate lymphocytosis include the following characteristics:

a. Infectious lymphocytosis occurs mainly in children.
   (1) The disorder is believed to be caused by viruses (e.g., coxsackievirus A and B6, echoviruses, adenovirus).
   (2) Symptoms include vomiting, fever, and abdominal discomfort.

b. Pertussis (i.e., whooping cough) is a childhood inflammatory reaction of the respiratory system.
   (1) Cause. The etiologic agent is the bacterium Bordetella pertussis.
   (2) Laboratory findings. Patients demonstrate a significant lymphocytosis as high as 30,000/mm³. Lymphocytosis is highest in the first 3 weeks of the disease, then decreases after 4 weeks. Lymphocytosis is caused by the release of lymphocytosis-promoting factor (LPF) from B. pertussis. LPF induces an acute release of lymphocytes from lymph nodes and also inhibits the migration of lymphocytes from the circulation into the lymphatics.
   (3) Morphologic characteristics. The lymphocytes in this disorder are small and mature.

c. IM (see Chapter 6, Section XIII B)
   (1) Serious complications of IM may require hospitalization.
     (a) AIHA is reported in 1% to 3% of patients, which is related to the development of an autoimmune anti-i.
     (b) Mild thrombocytopenia (i.e., approximately 5,000 to 100,000/mm³) is reported in 50% of patients who have IM.
     (c) Liver involvement can occur, which leads to mild jaundice and hepatitis.
     (d) Splenomegaly can also be a dangerous complication.
   (2) Recognizing hematologic features of IM can result in a rapid diagnosis.
     (a) Leukocytosis between 12,000/mm³ and 25,000/mm³ persists for the first 3 weeks of infection with a differential showing 60% to 90% lymphocytes.
     (b) A neutrophilia occurs during the first week of infection with a left shift, metamyelocytes, toxic granulation, and Döhle’s bodies.
     (c) Abundant atypical lymphocytes are found on the peripheral blood smear. These are active or transformed lymphocytes involved in an immune reaction. Atypical lymphocytes are not exclusive to IM and are also found in the following disorders:
       (i) Cytomegalovirus infections
       (ii) Toxoplasmosis
       (iii) Infectious hepatitis
       (iv) Viral pneumonia and mumps

d. Cytomegalovirus infection (CMV) demonstrates clinical and laboratory symptoms that are in some ways identical to those of IM.
   (1) Patients receiving massive blood transfusions are in a high-risk category for contracting CMV.
   (2) Presentation. Patients have a leukocytosis involving a lymphocytosis in which 20% or more of lymphocytes may be reactive.
(3) Serologic tests. Patients are negative for heterophil antibodies and Epstein-Barr virus (EBV) antibodies.
(4) Diagnosis is made by the demonstration of CMV antibodies by complement fixation or hemagglutination techniques.

e. Toxoplasmosis also produces clinical and laboratory symptoms similar to those of IM.
(1) Presentation. Patients have an absolute lymphocytosis and atypical lymphocytes on the peripheral blood smear.
(2) Serologic tests for EBV and heterophil antibodies are negative.
(3) Diagnosis is made by the demonstration of toxoplasmosis antibodies by the Sabin-Feldman dye test, fluorescent antibody, or hemagglutination.

f. Miscellaneous causes of lymphocytosis
(1) Syphilis
(2) Smallpox
(3) Para-aminosalicylic acid hypersensitivity
(4) Phenytoin (i.e., Dilantin) and Mesentoin hypersensitivity

4. Hypergammaglobulinemias are related disorders in which the levels of one or more serum immunoglobulins are increased above normal levels. Hypergammaglobulinemias may or may not be accompanied by an absolute lymphocytosis.

a. Multiple myeloma (MM) is a monoclonal gammopathy (i.e., an abnormality of only one B-cell clone) in which only one type of gamma-globulin is increased.
(1) MM involves a neoplastic proliferation of plasma cells primarily in the bone marrow. Plasma cell proliferation may be either nodular or diffuse.
(2) Clinical symptoms are found primarily in persons older than 40 years.
   (a) Bone pain is the most common symptom, and MM patients are often first seen with bone fractures.
   (b) Tumor growth in the marrow increases bone destruction, and the serum calcium level is high.
   (c) Patients have a high susceptibility to infection.
   (d) Renal insufficiency is also a common symptom with MM.
(3) The MM laboratory profile includes the following results:
   (a) Normocytic/normochromic anemia
   (b) Normoblasts on the peripheral blood smear
   (c) Rouleaux formation and increased erythrocyte sedimentation rate (ESR) (i.e., due to increase in serum globulins)
   (d) Possible shift to the left with metamyelocytes
   (e) Increased serum calcium level
   (f) Circulating plasma cells
   (g) An increase in one of the serum gamma-globulins as demonstrated by protein electrophoresis
(4) Bone marrow examination shows the presence of plasma cells varying from 1% to 90%.
(5) Serum immunoglobulins are increased in a variety of electrophoresis patterns among different patients. Serum protein electrophoresis usually shows a homogeneous band in the gamma or beta region of the electrophoretic tracing known as an “M-spot.”
   (a) Most patients with MM are hypergammaglobulin producers, but in 25% of patients, only the light chains of the globulin are produced (i.e., Bence Jones proteins) by the abnormal plasma cells. A serum hypergammaglobulinemia is found in these patients, because light chains are cleared by the kidney into the urine.
   (b) Half of reported MM patients show an increase in IgG only.
   (c) A monoclonal increase in IgA is found in approximately 20% of MM patients.
   (d) IgD is increased in approximately 1% of patients.
   (e) IgE is rarely found to demonstrate a monoclonal increase.
   (f) Proteinuria with Bence Jones proteins can also be demonstrated in more than 50% of MM patients.
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(6) Course, MM usually has a chronic course with the median survival approximately 3 years.
   (a) In 5% of MM patients, AMML develops as a secondary manifestation of the malignancy.
   (b) In some patients, plasmacytosis may be found in the blood and bone marrow, and the disease is then classified as plasma cell leukemia.

b. Waldenström’s macroglobulinemia (WM) is a hypergammaglobulinemia variant of chronic lymphocytic leukemia, in which there is a greater degree of maturation of the B lymphocytes into plasma cells.
   (1) WM is characterized by a generalized proliferation of B cells and plasma cells and an increase of monoclonal IgM in the serum that amounts to at least 15% of the total serum protein.
   (2) Clinical symptoms of WM are primarily found in individuals older than 40 years. Symptoms are caused by the cellular proliferation and increased blood viscosity caused by the increased IgM. Patients who have WM are commonly seen with the following symptoms:
      (a) Neurologic abnormalities
      (b) Renal insufficiency
      (c) Heart failure
      (d) Clotting abnormalities (e.g., DIC)
   (3) The laboratory profile includes the following results:
      (a) IgM in excess of 1.0 g/dL
      (b) Normocytic-normochromic anemia (i.e., occasionally hemolytic with a positive direct Coombs’ test)
      (c) Thrombocytopenia caused by IgM platelet clumping or pancytopenia caused by marrow infiltration
      (d) Lymphocytosis
      (e) Marked rouleaux and increased ESR
      (f) Bence Jones proteinuria in 10% of patients
   (4) The bone marrow often cannot be aspirated easily. A biopsy demonstrates an increase in small normal lymphocytes and plasma cells.

c. Heavy-chain diseases are disorders related to the production and excretion of the immunoglobulin heavy chains without the light chains.
   (1) Gamma heavy-chain disease (γ-HCD) resembles a malignant lymphoma rather than a myeloma.
      (a) The patient has a blood picture of anemia, leukopenia, thrombocytopenia, atypical lymphocytes, and plasma cells.
      (b) A broad serum protein “spike” is found in the beta-gamma region of the electrophoretic pattern, accompanied by serum hypogammaglobulinemia.
   (2) Alpha heavy-chain disease (α-HCD) is more common than γ-HCD and occurs in a younger age group.
      (a) Common symptoms include intestinal involvement with mal-absorption, diarrhea, and a massive lymphoplasmacytic infiltration in the intestinal mucosa.
      (b) The bone marrow and lymph nodes are not usually involved.
      (c) Protein electrophoresis is normal, but small amounts of α chain may be detected in serum and urine on immunoelectrophoresis.
   (3) Mu heavy-chain disease (μ-HCD) can be diagnosed only by a serum immunoelectrophoresis that demonstrates an increase in μ-chains.

5. Lymphoproliferative diseases
a. These disorders represent a group of clonal disorders originating from cells of the lymphoreticular system (Table 4–11).
   (1) When neoplastic cells involve mainly the bone marrow and blood, the disorder is known as a leukemia.
   (2) When the disease is limited mainly to lymph nodes or organs, the disease is known as a lymphoma.
   (3) Occasionally, a lymphoma develops into leukemia.
TABLE 4–11 Comparison and Differentiation of the Malignant Lymphoproliferative Disorders Based on Immunological Typing and Cytogenetic Abnormalities

<table>
<thead>
<tr>
<th>Disorder</th>
<th>Cell Type</th>
<th>CD Marker</th>
<th>Cytogenetic Abnormality</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chronic lymphocytic leukemia</td>
<td>T cell; 5%</td>
<td>CD2, CD3, CD8</td>
<td></td>
</tr>
<tr>
<td>PLL</td>
<td>B cell</td>
<td>sIg, CD19, CD20, CD22, CD24</td>
<td>t(11:14)</td>
</tr>
<tr>
<td>Acute lymphocytic leukemia</td>
<td>Early B; 70%</td>
<td>TdT, CD10, CD19, CD20</td>
<td>t(4:11)</td>
</tr>
<tr>
<td>Null cell</td>
<td>Pre-B</td>
<td>TdT, clg</td>
<td></td>
</tr>
<tr>
<td>T cell</td>
<td>Early T; 15%–20%</td>
<td>TdT, CD1, CD2</td>
<td></td>
</tr>
<tr>
<td>Burkitt type</td>
<td>Late B</td>
<td>sIg, CD19, CD20, CD22, CD24</td>
<td>t(4:11)</td>
</tr>
<tr>
<td>Lymphoma</td>
<td>B cell</td>
<td>sIg, CD19, CD20, CD24</td>
<td>t14:18)</td>
</tr>
<tr>
<td>NSCC</td>
<td>Late B cell</td>
<td>sIg, CD19, CD20, CD24</td>
<td>t(8 or 2, or 22:14)</td>
</tr>
<tr>
<td>SLL, DLCL</td>
<td>B cell</td>
<td>sIg, CD19, CD20, CD24</td>
<td>t(11:14)</td>
</tr>
<tr>
<td>TCL</td>
<td>Late T cell</td>
<td>CD2, CD3, CD4, CD5</td>
<td>t(7;14, or 11 or 9)</td>
</tr>
</tbody>
</table>

CALLA = common acute lymphocytic leukemia antigen; CD = cluster designation; clg = cytoplasmic immunoglobulin; DLCL = diffuse large-cell lymphoma; HCL = hairy cell leukemia; NSCC = nodular small cleaved lymphoma; PLL = prolymphocytic leukemia; sIg = surface membrane immunoglobulin; SLL = small-cell lymphocytic lymphoma; TdT = terminal deoxynucleotidyl transferase; + = trisomy.

b. Chronic lymphocytic leukemia (CLL) is a slowly progressing clonal malignancy of lymphocytes in an arrested stage of maturation.

(1) The patient’s clinical profile includes the following features:
   (a) CLL is commonly seen in adults with a mean age of occurrence at 55 years.
   (b) The disorder is twice as common in men as compared with women.
   (c) Onset is slow, unrevealing, and is commonly discovered incidentally or only in the late stages of the disease.
   (d) Patients are seen with symptoms, such as weakness, fatigue, anorexia, weight loss, enlarged lymph nodes, and abdominal discomfort caused by liver and spleen enlargement.

(2) The laboratory profile of a patient who has CLL includes the following:
   (a) Patients have a leukocytosis ranging from 10,000 to 150,000/mm³ with 80% to 90% lymphocytes persistent over a period of weeks to months.
   (b) Smudge cells are commonly found on blood smears.
   (c) Lymphocytes in patients with CLL have a characteristic morphology.
      (i) The nuclear chromatin is coarsely condensed.
      (ii) Nucleoli may be demonstrable.
      (iii) Lymphocytes show minimal size and shape variation.
      (iv) The cytoplasm is small to moderate in amount.
      (d) Occasionally, immature lymphocytes (i.e., usually fewer than 10%) may be found on the peripheral blood smear.
   (e) Patients who have CLL do not usually have anemia or thrombocytopenia in early stages. As lymphocyte proliferation replaces the marrow with leukemic cells, production of other cell lines may suffer, and the symptoms will appear.
   (f) AIHA develops in 10% of patients who have CLL. The patient’s blood smear demonstrates spherocytes and reticulocytosis.
   (g) Hypogammaglobulinemia may also be present because of qualitative defects of the leukemic lymphocytes.

(3) A marrow aspirate commonly shows an increase in morphologically mature lymphocytes.
   (a) If AIHA is present, then a marrow picture of increased erythropoiesis is predominant.
   (b) In later stages of CLL, the lymphocytes overrun the marrow, replacing the other hematopoietic tissues.

(4) Ninety-five percent of CLL patients immunologically type as having a B-cell leukemia.
(a) **Immunologic cell-marker** assays for lymphocytic membrane receptors, surface antigens, and enzymes are useful in differentiating between lymphocytic leukemias. **Cluster designation markers** for B lymphocytes include the following:

(i) **CD5**: positive in B-lymphocyte CLLs and some lymphomas

(ii) **CD22**: positive for late B cells and hairy-cell leukemia

(iii) **CD24**: positive in all stages of B-lymphocyte development (i.e., Pan-B)

(b) **Surface IgM** can be commonly detected but is weak.

(c) In most cases of B-cell leukemia, the ability of the lymphocytes to form spontaneous rosettes with mouse RBCs can be demonstrated (i.e., M rosettes).

(5) Five percent of CLL patients have a T-cell leukemia.

(a) Leukemic T lymphocytes may have a cleft-or clover-shaped nucleus (i.e., Sézary cells).

(b) **Skin involvement** and lymphocyte infiltration occur in 60% of T-cell CLL patients.

(c) **Immunologic markers** for T-cell leukemia include the following:

(i) **CD2**: E-rosette marker (i.e., sheep RBCs)

(ii) **CD3**: positive for T-lymphocyte CLL, T-cell prolymphocytic leukemia, and infectious mononucleosis

(iii) **CD8**: positive for T-lymphocyte CLL

(6) Staging systems have been devised to help categorize patients into prognostic groups.

(a) **Criteria** for staging include lymphadenopathy, anemia, hepatosplenomegaly, and thrombocytopenia.

(b) The absence of these symptoms indicates a good prognosis with a long survival (i.e., 20 years).

(c) With these symptoms, the patient’s survival can be up to 5 or 6 years with treatment.

(7) Treatment of CLL includes one or a combination of the following agents:

(a) Chemotherapy with alkylating drugs

(b) Glucocorticoid administration

(c) Radiotherapy

(8) Treatment can relieve symptoms and improve life expectancy and blood counts. However, hypogammaglobulinemia is often not corrected with treatment, and patients can succumb to infection.

c. Several clinical variations of CLL can be found.

(1) **Hairy-cell leukemia** (leukemic reticuloendotheliosis) is a rare form of CLL found four times more in men than women. The mean age of occurrence is 50 years.

(a) The onset of the disease is slow and is characterized by proliferation of abnormal lymphocytes in the secondary lymphoid organs.

(b) **Splenomegaly** is a common physical finding.

(c) The **laboratory profile** includes the following:

(i) Pancytopenia, or a depression of two cell lines, is common.

(ii) The peripheral blood smear may have variable numbers of “hairy-cell” lymphocytes. These lymphocytes are medium-sized cells with a round, oval, notched, or dumbbell-shaped nucleus. The cytoplasm of these lymphocytes is moderate in amount with numerous hair-like projections and frayed borders.

(iii) Hairy-cell lymphocytes contain an acid phosphatase resistant to inhibition by L-tartarate, resulting in a positive TRAP stain.

(iv) Lymphocytes in hairy-cell leukemia are usually B cells that demonstrate strong surface membrane immunoglobulin (sIg) and a positive reaction for common B-cell markers, such as CD19; CD20; CD22; CD24; and CD25 (i.e., IL2 receptor), which is unique to patients who have hairy-cell leukemia.

(d) The median survival of patients is 5 to 6 years.
Lymphosarcoma cell leukemia is a lymphocytic lymphoma that has transformed into a leukemic phase with the invasion of the marrow and blood of leukemic lymphocytes.

(a) The clinical course is more aggressive than in common B-cell CLL.
(b) Morphologic differences. Leukemic lymphocytes are B cells, but are morphologically different than those cells seen in regular B-cell CLL.
   (i) Nuclear chromatin is smoother with an oval or notched nucleus.
   (ii) Distinct nucleoli are easily seen in malignant lymphocytes.
   (iii) A lower N:C ratio is found in these lymphocytes when compared with the N:C ratio in regular B-cell CLL.
(iv) Lymphocytes demonstrate a strong expression of slg.

3. Prolymphocytic leukemia (PLL) is a variation of CLL in which there is a high number of morphologically immature larger lymphocytes, which appear as prolymphocytes.
   (a) This variation is typically seen in older men (i.e., older than 50 years).
   (b) Is characterized by a lymphocytosis up to 150,000/mm³.
   (c) Lymphocytes are the B-cell type.
   (d) Prolymphocytes can be distinguished by their smooth nuclear chromatin and large nucleoli.
   (e) Patients commonly have a massive splenomegaly, but their lymph nodes are not enlarged.
   (f) The prognosis is usually subacute and more resistant to treatment than is common CLL. Patients have a mean survival of 1 year.
   (g) PLL prolymphocytes commonly type positive with the membrane markers CD19, CD20, CD22, CD24, and strong expression of slg.

Acute lymphocytic leukemia (ALL) is a rapidly progressing clonal malignancy of early immature lymphocytes in an arrested stage of maturation.

(1) The clinical profile of ALL includes the following features:
   (a) ALL is mainly a disease of childhood with a peak incidence at 4 years.
      The second peak of incidence (i.e., bimodal distribution) is in young adults between 20 and 40 years.
   (b) Onset of leukemia is sudden with symptoms of anemia, bleeding, fever, and fatigue.
   (c) The spleen, liver, and lymph nodes are commonly enlarged.

(2) The laboratory profile of ALL includes the following results:
   (a) A normocytic-normochromic anemia is common.
   (b) Normoblasts are found on the peripheral blood smear.
   (c) The leukocyte count shows three patterns. The WBC count is occasionally very high (i.e., >100,000/mm³); often is slightly elevated; but most often is normal or decreased.
   (d) The predominant cell is the lymphoblast or immature lymphocyte.
   (e) The serum level of uric acid is often elevated.
   (f) A small percent of ALL patients are positive for the Ph₁ chromosome.
   (g) Immunodeficiency or hypogammaglobulinemia is not as common as in CLL.

(3) A marrow aspirate commonly shows diffuse infiltration of lymphoblasts.

(4) Acute lymphocytic leukemias are classified by the FAB group as L1, L2, and L3 (Table 4-12).
   (a) Approximately 70% of children with ALL are Type L1, and 27% are Type L2. The remaining 3% are Type L3.
   (b) The L2 type of ALL is the most common type of adult ALL.
   (c) L3 represents the B-cell Burkitt’s lymphoma type of ALL with vacuolated lymphoblasts.

(5) The diagnosis of ALL cannot be made with complete certainty until cytochemical staining procedures have been performed to distinguish the lymphoblasts and lymphocytes from positively reacting cells of AML.
   (a) ALL blasts are negative for Sudan black B, peroxidase, and naphthyl AS-D chloroacetate esterase.
### Table 4–12 Differentiation of Acute Lymphocytic Leukemia (ALL) Based on FAB Morphologic Classification and Immunologic Subtype

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>L1</th>
<th>L2</th>
<th>L3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell size</td>
<td>Homogeneous population of small blasts</td>
<td>Heterogeneous population of large blasts</td>
<td>Homogeneous population of large blasts</td>
</tr>
<tr>
<td>Nucleus</td>
<td>Homogeneous regular shape with slightly clumped chromatin; faint nucleoli</td>
<td>Variable shape with smooth chromatin; one or more large nucleoli</td>
<td>Regular shape with dense-fine homogeneous chromatin; one or more prominent nucleoli</td>
</tr>
<tr>
<td>Cytoplasm</td>
<td>High N:C ratio; slight-to-moderate basophilia</td>
<td>Moderate N:C ratio; moderate-to-deep basophilia</td>
<td>Moderate-to-low N:C ratio; deep basophilia with obvious vacuolation</td>
</tr>
<tr>
<td>Patient age</td>
<td>70% of childhood ALL</td>
<td>TdT</td>
<td>70% of adult ALL</td>
</tr>
<tr>
<td>Immunologic markers</td>
<td>CALLA (CD10); TdT, CD19, CD20</td>
<td>TdT</td>
<td>CALLA, CD19, CD20, CD20, CD24</td>
</tr>
</tbody>
</table>

**Immunologic markers**

- **CALLA** (common acute lymphocytic leukemia antigen, CD = cluster designation, FAB = French-American-British, N:C = nucleus:cytoplasm, sIg = surface membrane immunoglobulin, TdT = terminal deoxynucleotidyl transferase.

(b) The acid phosphatase reaction is positive in the 20% of patients whose ALL is of the T-lymphocyte type.

(c) Terminal deoxynucleotidy transferase (TdT) is an intracellular DNA polymerase that is present in T and B lymphoblasts.

(i) TdT is weakly positive in pre-B cells.

(ii) TdT is strongly positive in 60% to 85% of T-cell thymocytes.

(iii) Mature peripheral T cells and mature B cells are TdT negative.

(iv) TdT is measured with an immunofluorescence assay that uses a specific antibody against TdT prepared in rabbits.

(v) This assay is used in the diagnosis of ALL, lymphoblastic lymphoma, and the blast phase of CML. Lymphoblasts in 90% of ALL patients are TdT positive, but blasts in only 5% of nonlymphocytic leukemias (e.g., CML) stain positive for TdT.

(6) There are several functional subclasses of ALL based upon immunologic membrane surface markers.

(a) Common ALL antigen (CALLA), which is also CD10, is the most common subtype.

(i) Lymphocytes do not show surface features of B or T cells on blast membranes.

(ii) This subtype is the most common form of ALL in children and morphologically, lymphocytes are of the FAB classification of L1.

(b) Null-cell ALL is a proliferation of lymphocytes that type negative for T-cell, B-cell, and CALLA surface antigens.

(i) Includes a smaller proportion of children and more adults.

(ii) Null-cell ALL is thought to be a pre-B-cell leukemia.

(c) T-cell ALL accounts for 10% to 20% of all patients who have ALL.

(i) This functional subtype occurs mainly in boys.

(ii) Patients usually show a high leukocyte count pattern and have a poor prognosis.

(iii) T-cell ALL is characterized by a high frequency of mediastinal tumor, skin, and CNS involvement.

(iv) Malignant lymphoblasts demonstrate T-cell markers such as CD2 (i.e., E rosettes); TdT; and CD1.

(d) B-cell ALL is the rarest of subtypes, and it corresponds to the FAB classification Burkitt’s Type L3.

(i) Patients who have Type L3 generally have a poor prognosis.

(ii) L3 can be diagnosed by demonstration of B-cell typical surface Ig receptors.

(iii) This disorder is thought to be a memory-cell leukemia.

(7) Better prognosis is seen in female patients, patients with lower WBC counts, and patients diagnosed with the FAB classification L1 subtype.
Treatment is now able to produce complete remission in 95% of children and in 50% to 70% of adults with ALL. Approximately 50% of children in remission show signs of being completely cured.

e. Lymphomas are a group of lymphoproliferative disorders in which there is neoplastic proliferation of an arrested stage of secondary lymphocyte maturation that usually begins in and involves mainly the lymph nodes. As the disease progresses, many other organs and tissues (e.g., spleen, liver, skin, marrow) can become invaded by malignant lymph. All lymphomas are categorized into either Hodgkin’s lymphoma or non-Hodgkin’s lymphoma.

(1) The general laboratory profile of a patient who has lymphoma includes the following results:
   (a) The WBC count may vary, depending on the progression of the malignancy.
      (i) Neutrophilia is seen only when lymph nodes are involved, but neutropenia persists when the bone marrow is involved.
      (ii) Most frequent are WBC counts from 12,000 to 25,000/mm³ with lymphophilia and monocytosis.
      (iii) Eosinophilia is found in 20% of patients who have lymphoma.
      (iv) Platelet count depends upon the extent of marrow involvement.
   (b) Severe Normochromic/normocytic anemia in 50% of patients.
   (c) The LAP level is elevated in the active phases of the disease.
   (d) Reed-Sternberg cells, which are the hallmark of Hodgkin’s lymphoma, may be found in the lymph nodes and marrow of a patient who has this disease.
      (i) These cells can be identified as giant binucleated or multi-nucleated cells with acidophilic nuclei.
      (ii) This cell type is thought to originate from the monocyte-macrophage cell line.
   (e) Bone marrow biopsy frequently reveals granulocytic hyperplasia with a shift to the left, slight monocytosis, and eosinophilia. Reed-Sternberg cells may be present, depending on the clinical subtype.

(2) The Hodgkin’s type of lymphoma has an increased frequency of occurrence in persons between the ages of 15 and 30 years and after 50 years.
   (a) The clinical subclassification and diagnosis of Hodgkin’s lymphoma is made histologically from a lymph node biopsy.
      (i) Lymphocytic-predominant Hodgkin’s lymphoma is most frequent in young men and localizes to the cervical lymph nodes. Lymph-node biopsy reveals numerous lymphocytes, no fibrosis or necrosis, and few Reed-Sternberg cells.
      (ii) Nodular-sclerosis Hodgkin’s lymphoma is a common variety most often discovered as a mediastinal mass in young women. Lymph-node biopsy is characterized by broad bands of collagen separating nodules of lymphoid tissue and the presence of lacunar cells, which are atypical histiocytes. Few Reed-Sternberg cells are found.
      (iii) The mixed type of Hodgkin’s lymphoma is characterized on lymph-node biopsy by a variety of all cell types (e.g., lymphocytes, plasma cells, eosinophils, histiocytes, and numerous Reed-Sternberg cells). Necrosis and fibrosis may also be present.
      (iv) The lymphocyte depletion type of Hodgkin’s lymphoma is the rarest type. Lymph-node biopsy reveals diffuse fibrosis and a lack of lymphoid growth. This type is accompanied by pancytopenia and lymphocytopenia, because there is a more frequent involvement of bone marrow.
   (b) Clinical staging of Hodgkin’s lymphoma is based on the extent of lymph node and systemic involvement.
      (i) Stage I of the disease is limited to lymph nodes in one or two anatomic regions on one side of the diaphragm.
      (ii) Stage II of the disease involves more than two regions of lymph node involvement on one side of the diaphragm.
Stage III of the disease involves two or more regions of node involvement on both sides of the diaphragm.

Stage IV of the disease involves bone marrow and other organs, in addition to lymph nodes. Cell-mediated immunity is defective by this stage.

(c) Treatment. Without treatment, few patients survive as long as 10 years. With modern treatment methods, 85% of patients diagnosed in Stages I and II can be cured. The cure rate is 50% for patients diagnosed in Stages III or IV. Treatment consists of long-term chemotherapy and localized radiotherapy for infected lymph nodes.

(3) Non-Hodgkin’s lymphomas are classified as a group of clonal proliferations of lymphocytes that have undergone malignant transformation at an arrested stage of their differentiation. Non-Hodgkin’s lymphomas involve mostly B lymphocytes and are reported mostly in middle-age and older patients.

(a) The Rappaport classification of non-Hodgkin’s lymphomas is based upon nodular and diffuse arrangement of malignant cells in a lymph-node biopsy, as well as on size characteristics of the predominating lymphocytes.

(b) Many subtypes of non-Hodgkin’s lymphoma are closely related to a defect in immune regulation.

(c) Some subtypes are believed to be virally related.

(i) Burkitt’s lymphoma, which is associated with EBV, has a strong affinity for B cells; this affinity causes B-cell proliferation. When the cytolytic killer T lymphocytes’ response is deficient, an unchecked B-cell proliferation can result in lymphoma.

(ii) Cutaneous T-cell lymphoma (i.e., Mycosis Fungoides) is an HTLV-1-promoted helper-inducer T-cell malignancy. This lymphoma is associated with a lymphocytosis of Sézary cells.

(d) Most patients who have non-Hodgkin’s lymphomas have associated chromosome abnormalities (Table 4–11).

(i) Nodal small cleaved-cell lymphoma is associated with a translocation between the genes of Ig heavy chains found on chromosome 14 and an arm of chromosome 18.

(ii) Burkitt’s lymphoma is associated with a translocation between chromosomes 8 and 2 or 22 and 14.

(iii) Small lymphocytic lymphoma and diffuse large-cell lymphoma are associated with a translocation between chromosomes 11 and 14.

(iv) Cutaneous T-cell lymphoma is associated with a translocation between chromosomes 7 and 14 or 11 and 9.

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CHAPTER 5
Immunology and Serology

WADE REDMAN, MBA, MT (ASCP) DLM AND JOEL HUBBARD, PhD, MT (ASCP)

I. INTRODUCTION

A. Antigens and haptens. An antigen is a substance that stimulates antibody formation and has the ability to bind to an antibody. A hapten is a low-molecular-weight, nonantigenic substance that, when combined with an antigen, changes the antigenic specificity of that antigen.

B. An antibody is a glycoprotein substance (immunoglobulin) that is produced by B lymphocytes in response to an antigen.

1. Antibodies may be monoclonal or polyclonal. Monoclonal antibodies are derived from a single B-cell clone and are produced as a single class of immunoglobulin with specificity unique to the antigenic stimulus.

2. Polyclonal antibodies are produced as different classes of immunoglobulins by many B-cell clones in response to an antigen.

3. Antibodies produced in response to antigens from another species are called heteroantibodies or xenoantibodies.

4. Alloantibodies are formed in response to antigens from individuals of the same species.

5. Autoantibodies are produced by the body’s immune system against “self” antigens. They function by facilitating phagocytosis and microbial killing and by neutralizing toxic substances. They also combine with antigens on cell surfaces, which results in either intravascular or extravascular destruction of the target cells.

C. Antigen-antibody reactions

1. An epitope is the part of an antigen that reacts specifically with an antibody or T-cell receptor (Figure 5–1).

2. Agglutination is the clumping of particulate antigens by antibodies specific for the antigens.

3. Affinity is the tendency that an epitope has for combining with the antigen-binding site on an antibody molecule.

4. Avidity is the strength of the bond between the antigen and the antibody.

5. Sensitivity is the smallest amount of antigen or antibody that can be detected.

6. Specificity is the ability of an antibody to bind to an antigen with complementary determinants and not to an antigen with dissimilar determinants.
Figure 5–1 Basic immunoglobulin (lg) molecule. Four polypeptide chains are linked covalently by disulfide bonds (S–S). The loops correspond to domains within each chain. V = variable domain; C = constant domain; L = light chain; H = heavy chain; CHO = carbohydrate side chain. The inset shows the hypervariable complementarity-determining regions as shaded areas. Similar hypervariable regions are found in the alpha and beta chains of the T-cell receptor for antigen. (Reprinted from Hyde RM. *Immunology*, 3rd ed. Baltimore: Williams & Wilkins; 1995:40 with permission.)
D. Immunoglobulins are glycoprotein substances secreted by antigen-stimulated B cells. All classes of immunoglobulins share the same basic structure: two heavy chains and two light chains joined by varying numbers of disulfide bonds (see Figure 5–1). Heavy chains determine the isotype, or class, of immunoglobulin. There are five immunoglobulin classes: IgA, IgD, IgE, IgG, and IgM (Table 5–1). Each molecule of immunoglobulin has either two kappa light chains or two lambda light chains. (Web Color Images 5–1 and 5–2)

1. IgA exists as a monomer in serum and as a dimer in body secretions. It is the predominant immunoglobulin in secretions such as tears, saliva, sweat, breast milk, and respiratory tract, genital, and intestinal secretions. IgA binds antigens and prevents their adherence to mucous membranes to keep them from invading the body. It also confers immunity from mother to infant through breast milk. The normal range for serum IgA is 77 to 400 mg/dL.

2. IgD exists as a monomer, and its function is unknown. Normal serum values of IgD are 3 to 5 mg/dL.

3. IgE exists in serum as a monomer. It binds to crystallizable fragment (Fc) receptors on mast cells and basophils and is elevated during parasitic infections and Type I allergic reactions. Normal serum values for IgE are 17 to 450 mg/dL.

4. IgG is the predominant immunoglobulin in the adult. There are four subclasses of IgG: IgG1, IgG2, IgG3, and IgG4. IgG binds complement, has roles in opsonization and antibody-dependent cellular cytotoxicity (ADCC), and neutralizes toxins. It is also the only immunoglobulin that crosses the placental barrier, thus transferring immunity from mother to infant through breast milk. IgG precipitates and agglutinates in vitro. Normal serum values for IgG are 591 to 1,965 mg/dL.

5. IgM is the largest of the immunoglobulins, existing as a pentamer (Figure 5–2). It binds complement, neutralizes toxins, and agglutinates antigens in vitro. IgM is the first immunoglobulin to be produced after exposure to an antigen. Normal serum values are 50 to 311 mg/dL.

E. Complement. The complement system is a group of proteins synthesized in mononuclear phagocytes, hepatocytes, fibroblasts, and some endothelial cells. The native precursor components are numbered from 1 to 9 with subcomponents of the proteins receiving the letters a to e as they are cleaved.

1. Function. Complement proteins are involved in the disruption of microbial cell walls, inflammation mediation, regulation of phagocytic activity, and the metabolism of immune complexes. Complement can be activated through either the classic pathway, the alternate pathway, or the mannan-binding lectin (MBL) pathway.

   a. The classic pathway requires IgG or IgM for activation. Table 5–2 and Web Color Image 5–3 both describe the classic pathway of complement activation.

   b. The alternate pathway does not require specific antibody for activation, but is instead triggered by polysaccharides and lipopolysaccharides on the surfaces of certain target cells.

   c. The mannan-binding lectin (MBL) pathway is activated by binding of mannan-binding lectin (MBL) to mannose-containing residues of glycoproteins on certain microorganisms. MBL-associated serine protease (MASP) binds to MBL, which leads to activation of components C2, C4, and C3. This pathway is very similar to
Figure 5–2: Structural models of IgM and secretory IgA (slgA). IgM has a pentameric structure linked by the J chain at the Fc fragment. The slgA molecule has a dimeric structure, plus joining (J) chain, plus secretory component. Shown is the dominant IgA2 subclass. (Reprinted from Hyde RM. Immunology, 3rd ed. Baltimore: Williams & Wilkins; 1995:47 with permission.)

the classical pathway except that it differs in the initial recognition and activation steps. (Web-Color Image 5–4)

d. Anaphylotoxins are the small fragments (C3a, C4a, C5a), which attract and activate different types of leukocytes (Web Color Image 5–5). Anaphylotoxins increase vascular permeability, can cause contraction of smooth muscle, induce the release of histamine from basophils and mast cells, and draw in additional cells to the site of infection to help eliminate the microbes. C5a is the most potent, followed by C3a and C4a.

2. Membrane attack complex. Although these two pathways are separate and function independently, they converge at the C5 reaction, and the reactions from C5 to C9 are

<table>
<thead>
<tr>
<th>Table 5–2 Classic Pathway of Complement Activation</th>
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<tbody>
<tr>
<td><strong>Activation of C1</strong></td>
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<tr>
<td><strong>Activation of C4</strong></td>
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<tr>
<td><strong>Activation of C2</strong></td>
</tr>
<tr>
<td><strong>Activation of C3</strong></td>
</tr>
<tr>
<td><strong>Membrane attack complex</strong></td>
</tr>
<tr>
<td><strong>Activation of C5, C6, and C7</strong></td>
</tr>
<tr>
<td><strong>Membrane attack complex</strong></td>
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<tr>
<td><strong>Membrane attack complex</strong></td>
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<tr>
<td><strong>Activation of C8 and C9</strong></td>
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<tr>
<td><strong>Resulting channels allow water to enter the cell; Lysis occurs</strong></td>
</tr>
</tbody>
</table>
common to both pathways. These last components compose the common pathway and are also called the membrane attack complex. The C567 complex acts on C8 and C9. As C8 and C9 are inserted into the cell membrane, the resulting lesions allow the rapid passage of ions, and the cell lyses from osmotic pressure changes.

II. CELLS AND TISSUES OF THE IMMUNE SYSTEM. There are several cell types (Web Color Image 5–6) and body tissues that work together in the immune system. Many surface antigens or surface markers have been identified that help to characterize these cells according to their function. These markers are referred to as clusters of differentiation (CD). An international nomenclature system has been developed to standardize the CD numbers. Table 5–3 lists examples of CD markers.

A. T lymphocytes
1. T lymphocytes are derived from cells in the bone marrow. This subset of lymphocytes migrates to the thymus, where they mature and acquire and express certain surface antigens. T lymphocytes are responsible for cell-mediated immune responses. They exist in several subpopulations with specific functions.
   a. T helper cells (TH) are CD4 positive and produce the lymphokines interleukin-2 (IL-2), interleukin-3 (IL-3), granulocyte-monocyte colony-stimulating factor (GM-CSF), and gamma interferon (IFN-γ). They aid in B-cell differentiation, and they stimulate other T-cell populations.
   b. T suppressor cells (Ts) are CD8 positive and produce factors that inhibit the action of other T cells.
   c. Most cytotoxic T cells (Tc) are CD8 positive. They secrete lymphotoxins and release perforins, which destroy cells recognized as foreign.
   d. Delayed-type hypersensitivity T cells (T DTH) are CD4 positive. They secrete macrophage chemotaxin and macrophage migration inhibition factor (MIF).
2. If a T lymphocyte has surface receptors for a specific antigen, contact with that antigen stimulates blastic transformation. The resulting large lymphocyte then subdivides to produce two expanded populations of T cells: small lymphocytes and medium lymphocytes, both of which have the same antigenic specificity. Several mitogenic substances

| Table 5–3: Clusters of Differentiation (CD) |
| CD | Leukocytes |
| CD2 | T lymphocytes |
| CD4 | T lymphocytes |
| CD8 | T lymphocytes |
| CD10 | Pre-B lymphocytes, common ALL |
| CD12 | Monocytes, granulocytes, platelets |
| CD15 | Granulocytes |
| CD19 | B lymphocytes |
| CD24 | B lymphocytes, granulocytes |
| CD27 | T lymphocytes, plasma cells |
| CD33 | Myeloid leukemia |
| CD34 | Hemopoietic stem cells |
| CD39 | B lymphocytes, macrophages |
| CD41a | Glycoprotein Ib/IIa |
| CD45 | Leukocytes |
| CD47 | Leukocytes, platelets |
| CD68 | Macrophages |
| CD69 | Activated Lymphocytes |
| CD70 | Reed-Sternberg cells |
| CD77 | Activated B cells |

ALL = acute lymphocytic leukemia.
have been found to stimulate T cells and, thus, can be used in laboratory evaluation of T-cell function, including:

a. Concanavalin A (ConA)
b. Phytohemagglutinin A (PHA)
c. Pokeweed mitogen (PWM)

B. B lymphocytes

1. Mature B lymphocytes secrete immunoglobulin. These cells develop in the bone marrow in adults and are later localized also in the spleen and lymph nodes. There are several identifiable stages of B-cell maturation.

   a. Both T and B stem cells have terminal deoxynucleotidyl transferase (TdT). Stem B cells also have immune response (Ir) gene products, which are major histocompatibility complex (MHC) proteins.

   b. The pre-B cell is characterized by immunoglobulin gene rearrangement and the appearance of heavy chains in the cell's cytoplasm.

   c. In the immature B cell, light chain genes are rearranged, and the light chains appear in the cell's cytoplasm. Coupled with the heavy chains, the light chains form IgM, which is confined to the cell surface (sIgM).

2. In addition to antigens and T-cell products, B cells also respond to a variety of other mitogens including:
   a. Lipopolysaccharides found on the cell surface of gram-negative bacteria
   b. Protein A found on the surface of Staphylococcus aureus
   c. PHA
d. PWM
e. Phorbol myristate
f. Anti-IgM antibody

C. Non-T, non-B lymphocytes have neither T-cell nor B-cell markers and have been classified as killer cells (K), natural killer cells (NK), and lymphokine-activated killer cells (LAK). It is unclear as to whether they represent separate populations or are the same cells with different functions.

1. K cells express surface immunoglobulin receptors and lyse target cells by ADCC.
2. NK cells play a role in tumor host defense, because they have the ability to recognize and destroy tumor cells.
3. LAK cells use IL-2 to help lyse tumor cells.

D. Phagocytes play a major role in the immune system.

1. Polymorphonuclear leukocytes (PMNs) include eosinophils, basophils, and neutrophils.

   a. Eosinophil granules contain antimicrobial agents.

   b. Basophil granules contain histamine and heparin, which play a role in anaphylactic reactions.

   c. The two major populations of neutrophil granules are primary and secondary granules.

      (1) Primary granules contain myeloperoxidase (MPO) and lysozyme, which are important bactericidal agents.

      (2) Secondary granules also contain lysozyme. Neutrophils have chemotactic receptors, which allow them to respond to chemotaxins produced at sites of inflammation.

         (a) Chemotaxins commonly involved in this process include the C5a component of complement, formyl peptides found in bacteria, and arachidonic acid metabolites released from damaged cell membranes.

         (b) The ingestion of bacteria and other foreign particles by these cells is enhanced if the particles are coated with immunoglobulin or the C3b component of complement (opsonization).

2. Monocytes and macrophages

   a. Function. As circulating monocytes respond to chemotaxins, they leave the circulation and enter the tissues, where they are converted to macrophages. These cells are important in antigen processing and antigen presentation.
b. Surface receptors. Macrophages have several different surface receptors, including the integrin Mac 1-CR3, which binds the C3b component of complement; Fc receptors, which bind immunoglobulin; CR1, which binds C3b and C4b; and Class II MHC receptors.

c. Secreted products. Macrophages secrete products that assist in the immune response:

1. Interleukin 1 (IL-1) stimulates T-cell growth.
2. Interleukin 6 (IL-6) stimulates B cells.
3. Tumor necrosis factor (TNF\(\alpha\)) has antitumor and antibacterial activity and stimulates production of IL-1 and interferon.
4. Endothelial leukocyte adhesion molecule (ELAM-1) plays a role in diapedesis (migration of cells into tissues from the circulation).

E. Other cells involved in antigen presentation are:

1. Dendritic cells
2. Langerhans cells
3. B lymphocytes

F. Lymphoid tissues are found throughout the entire body.

1. Distribution
   a. Spleen
   b. Thymus
   c. Thoracic duct
   d. Lymph nodes
   e. Bone marrow
   f. Peyer’s patches
   g. Tonsils
   h. Appendix

2. Concentration. Although both T and B lymphocytes can be found in all lymphoid tissues, the highest concentration of T cells is found in the thymus. The highest concentration of B cells is found in the bone marrow.

III. IMMUNITY may be natural or acquired.

A. Natural immunity is present at birth and provides protection against disease and aids in recovery from disease. However, it also provides the basis for organ rejection after transplantation. Factors involved in natural immunity include:

1. Physical barriers, such as skin and mucous membranes
2. Genetically controlled susceptibility and nonsusceptibility to certain diseases
3. Inflammation, which involves a vascular response and a cellular response by phagocytic cells
4. Acute-phase plasma proteins, such as C-reactive protein, haptoglobin, and fibrinogen, which are produced in response to injury and aid in wound healing

B. Acquired or specific immunity results when immunologic memory and antibody specific to a foreign antigen develop in response to the antigen. Acquired immunity may be active (through immunization or disease) or passive (through transplacental transfer). This type of immunity involves both cell-mediated and humoral immune responses.

IV. IMMUNE RESPONSE (IR). The IR may be roughly divided into two components, cell-mediated and humoral. Although most IR contain elements of both, certain antigens elicit a strong humoral response with little cell-mediated involvement, and other antigens elicit primarily a cell-mediated response.

A. Humoral immunity involves immunoglobulin (antibody) production by B lymphocytes. Complement can also be considered a humoral component because it can be activated by immunoglobulin. The humoral response occurs in three phases:
1. **Antigen elimination.** This phase is accomplished by phagocytosis. Most injected antigen is removed within minutes, but complete removal may take months or years.

2. **The primary response.** After exposure to an antigen, there is a latent period of approximately 5 to 15 days before antibody appears in the serum. The antibody titer increases, plateaus, then decreases. IgM is the first immunoglobulin to appear. Although a small amount of IgG is made later, the majority of immunoglobulin produced during a primary response is IgM.

3. **The secondary response.** A second or any subsequent exposure to the same antigen elicits a secondary response. This time, there is a rapid antibody response, usually within 2 to 4 days after antigen exposure. IgG is the predominant immunoglobulin. The circulating antibody titer is much higher and lasts longer than that seen in the primary response.

**B. Cell-mediated immunity** is especially important in viral and fungal infections and in infections caused by acid-fast bacilli (e.g., tuberculosis, Hansen’s disease).

1. **Macrophages, Tc, and NK cells** play a role in cell-mediated immunity (see Sections II A1 c, II C2, and II D2).

2. **ADCC.** Cells with cytolytic activity and Fc receptors, especially NK cells, are able to directly lyse antibody-coated (usually IgG) target cells.

3. **Cytokines** are protein messengers produced by cells. Many play a role in cell-mediated immunity. (Web Color Image 5–7)
   - **Lymphokines** are produced primarily by activated T lymphocytes and include:
     1. IL-2
     2. IL-3
     3. IL-4
     4. GM-CSF
     5. B-cell growth factor 2
     6. Macrophage activating factor
     7. MIF
     8. IFN-γ
   - **Monokines** are produced by monocytes and include:
     1. IL-1
     2. TNFα

**V. MAJOR HISTOCOMPATIBILITY COMPLEX**

**A.** In transplantation studies, MHC gene products were identified as responsible for graft rejection. Likewise, studies on responses to antigens, demonstrated that MHC gene products were found to control immune responses. It was determined that antigen-specific T cells recognize portions of protein antigens that are bound noncovalently to MHC gene products.

**B.** The Major histocompatibility complex is found on human chromosome 6.

1. Have a role in intercellular recognition and discrimination of self/nonself.
2. Have a role in development of humoral and cell-mediated immune responses.
3. One’s set of MHC genes influences the repertoire of antigens to which that person’s T\(_D\) and T\(_C\) cells can respond, therefore, one’s MHC plays a role in susceptibility to disease and autoimmunity.

**C.** Organization of MHC is summarized in Table 5–4.

1. **Class I MHC** are glycoproteins found on most nucleated cells. Class I molecules present antigenic peptides to the T\(_C\).
2. **Class II MHC** are glycoproteins found on antigen presenting cells (APC).
3. **Class III MHC** consists of secreted proteins with immune function.
4. **Inheritance**
   - a. MHC alleles are inherited as two sets. One set from each parent.
   - b. One set is the haplotype.
Table 5–4 Human HLA Arrangement

<table>
<thead>
<tr>
<th>Complex</th>
<th>MHC Class</th>
<th>II</th>
<th>III</th>
<th>I</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gene Products</td>
<td>DP</td>
<td>DQ</td>
<td>DR</td>
<td>Complement Proteins</td>
</tr>
<tr>
<td></td>
<td>αβ</td>
<td>αβ</td>
<td>αβ</td>
<td>TNF-α</td>
</tr>
<tr>
<td>Gene Region</td>
<td>DP</td>
<td>DQ</td>
<td>DR</td>
<td>CYP21, C4B, C4A, BF, C2, HSP70, TNF</td>
</tr>
</tbody>
</table>

e. A heterozygous human inherits one paternal and one maternal haplotype, each containing three Class-I (B, C and A) and three Class II (DP, DQ and DR) loci.
d. A heterozygous individual will inherit a maximum of six Class I specificities.
e. Similarly, the individual will also inherit DP and DQ genes and express both parental antigens.

D. Function and characteristics of MHC is summarized in Table 5–5.

1. Class I MHC:
a. Class I molecules are found on virtually all body tissue cells.
b. Platelets express primarily Class I HLA-A and HLA-B antigens.
c. Specific for antigens of 8 to 10 amino acid peptide length
d. Peptide Interactions:
   (1) Presentation of peptides to CD8+ T-cells
   (2) Endogenous intracellular proteins digested to peptides in cytosol of cell
   (3) Transported to endoplasmic reticulum where peptides are associated with Class I MHC
   (4) Peptides of suitable length (∼8–18 amino acids) are specifically transported across the ER membrane.

2. Class II MHC:
a. Class II molecules are found on B lymphocytes, activated T lymphocytes, monocytes, macrophages, dendritic cells, early hematopoietic cells, and some tumor cells.
b. Peptide interactions:
   (1) Class II molecules present peptides to CD4+ T cells,
   (2) Peptides are exogenous in origin and processed through the endocytic pathway.
   (3) Peptide interactions are membrane bound proteins.
   (4) Specific for antigens of 13 to 18 amino acid peptide length

3. Class III MHC molecules consist of secreted proteins with immune function.
a. Complement components C2, C4a, C4b, and factor B
b. Steroid enzymes 21-hydroxylase enzyme A and 21-hydroxylase enzyme B

Table 5–5 Comparison of MHC Types

<table>
<thead>
<tr>
<th>MHC-I</th>
<th>MHC-II</th>
<th>MHC-III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gene Products</td>
<td>HLA-A</td>
<td>DP</td>
</tr>
<tr>
<td></td>
<td>HLA-B</td>
<td>DQ</td>
</tr>
<tr>
<td></td>
<td>HLA-C</td>
<td>DR</td>
</tr>
<tr>
<td>Cellular Expression</td>
<td>Platelets</td>
<td>Tα cells (species variable)</td>
</tr>
<tr>
<td>Tc-cells</td>
<td>B-cells</td>
<td>None</td>
</tr>
<tr>
<td>B-cells</td>
<td>Macrophages</td>
<td></td>
</tr>
<tr>
<td>Macrophages</td>
<td>Dendritic cells</td>
<td></td>
</tr>
<tr>
<td>Dendritic cells</td>
<td>Endothelial cells</td>
<td></td>
</tr>
<tr>
<td>Endothelial cells</td>
<td>Hepatocytes (weak)</td>
<td></td>
</tr>
<tr>
<td>Hepatocytes (weak)</td>
<td>8–10 amino acid peptides</td>
<td></td>
</tr>
<tr>
<td>8–10 amino acid peptides</td>
<td>CD8+ T-cells</td>
<td></td>
</tr>
<tr>
<td>CD8+ T-cells</td>
<td>13–18 amino acid peptides</td>
<td></td>
</tr>
<tr>
<td>Antigen Presentation</td>
<td>Endogenous – (endothelial reticulum)</td>
<td></td>
</tr>
<tr>
<td>Endogenous – (membrane receptor)</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>Antigen Specificity</td>
<td>13–18 amino acid peptides</td>
<td></td>
</tr>
<tr>
<td>Membrane Receptor</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cellular Location of Recognition</td>
<td>Endogenous – (endothelial reticulum)</td>
<td></td>
</tr>
<tr>
<td>8–10 amino acid peptides</td>
<td></td>
<td></td>
</tr>
<tr>
<td>13–18 amino acid peptides</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
c. Inflammatory proteins TNF-α and TNF-β.

d. Heat shock proteins such as HSP70.

e. These are not related structurally to the MHC-I and MHC-II molecules.

f. They have no role in antigen presentation.

g. They do play a role in the immune response.

E. Diversity of the human MHC locus

1. Class I MHC—known alleles; 60A \( \times \) 110B \( \times \) 40C = \( 2.6 \times 10^5 \)

2. Class II MHC—known alleles; 4DP \( \times \) 6DQ \( \times \) 488 DR = \( 1.2 \times 10^4 \)

3. Total possible combination; (2.6 \( \times \) \( 10^5 \))\( \times \)(1.2 \( \times \) \( 10^4 \)) = 3.12 \( \times \) \( 10^9 \)

F. Clinical significance of HLA

1. Organ transplant & bone marrow transplant will have an increased survival rate of the patient and a decreased risk of graft vs. host disease if HLA and MHC matching between donor and recipient is done.

2. Platelet transfusion matching between donor and recipient is useful to patients who are retracted to random donor platelets.

3. HLA compatibility exerts the strongest influence on long-term kidney survival after a transplant.

4. HLA identical donors for bone marrow transplantation to reduce the frequency of graft vs. host disease.

5. Paternity testing for exclusion or nonexclusion.

6. Disease association
   a. Association between HLA phenotype and some diseases
   b. HLA-B27 is found in >90% of patients with ankylosing spondylitis, but in only ~10% normal individuals.

G. HLA Testing

1. A heterozygous human inherits one paternal and one maternal haplotype, each containing three Class I (B, C and A) and three Class II (DP, DQ and DR) loci.

2. A heterozygous individual will inherit a maximum of six Class I specificities.

3. Similarly, the individual will also inherit DP and DQ genes and express both parental antigens.

4. The Class II MHC molecule consists of two chains (α and β), with some antigenic determinants (specificities) on each chain.
   a. DR α- and β-chains can associate in either cis (both from the same parent) or trans (one from each parent) combinations, an individual can have additional DR specificities.
   b. Also, there is more than one functional DR β-chain gene. Hence, many DR specificities can be found in any one individual.

5. A microcytotoxicity test demonstrates that if the antigen is present on the lymphocytes, addition of complement will cause them to become porous and unable to exclude the added dye.
   a. White blood cells from the potential donors and recipient are distributed into a series of wells on a microtiter plate, and then antibodies specific for various Class I and Class II MHC alleles are added to different wells.
   b. After incubation, complement is added to the wells and cytoxicity is assayed by the uptake or exclusion of various dyes by the cells.
   c. If the WBC expresses the MHC allele for which a particular monoclonal antibody is specific, the cells will lysed upon addition of complement, and these dead cells will take up a dye such as trypan blue.
   d. The reaction of donor and recipient cells with a single antibody directed against an HLA-A antigen.

6. Mixed-lymphocyte reaction (MLR) can be used to quantify the degree of class II MHC compatibility between potential donors and a recipient.
   a. Lymphocytes from potential donor that have been x-irradiated or treated with mitomycin C serve as the stimulator cells, and lymphocytes from the recipient serve as responder cells.
b. Proliferation of the recipient T cells, which indicates T cell activation, is measured by the uptake of thymidine into cell DNA.

c. The greater the Class II MHC differences between the donor and recipient cells, the more thymidine uptake will be observed in an MLR assay.

d. Intense proliferation of the recipient lymphocytes indicates a poor prognosis for graft survival.

e. The advantage of the MLR over microcytotoxicity typing is that it gives better indication of the degree of TH cells activation generated in response to the Class II MHC antigens of the potential graft.

VI. HYPERSENSITIVITY

is an enhanced immune reaction to an antigen. These immune reactions are classified into four different types, depending on their pathophysiology (Table 5–6). Types I, II, and III are humoral (i.e., antibody mediated), and type IV hypersensitivity reactions are cell-mediated.

A. Type I reactions are immediate-type hypersensitivities that can range from mild food allergies to anaphylactic shock. Antigens complex with IgE and attach to basophils or tissue mast cells, which results in the release of histamines and the synthesis of leukotrienes C4, D4, and E4 (Web Color Image 5–8). Although Type I reactions can be systemic, most are localized. Examples include:

1. Urticaria (hives)
2. Hay fever
3. Bronchial asthma
4. Food allergies

B. Type II reactions are cytotoxic responses. In these reactions, complement-fixing IgG or IgM antibodies are directed against cellular or tissue antigens such as those found on the surface of white blood cells (WBCs) and platelets (Web Color Image 5–9). Examples include:

1. Hemolytic transfusion reactions (HTR) (Web Color Image 5–10)
2. Hemolytic disease of the newborn (HDN)
3. Immune thrombocytopenia
4. Certain drug allergies

C. Type III reactions are immune complex reactions. IgG and IgM antibodies form soluble immune complexes with antigens. These complexes may be deposited in extravascular tissues, which results in infiltration by neutrophils and local tissue damage. Complement is also activated and contributes to the inflammatory response. Examples include:

1. Immune glomerulonephritis
2. Serum sickness
3. Arthus reaction (Web Color Image 5–11)

<table>
<thead>
<tr>
<th>Type</th>
<th>Mechanism</th>
<th>Example</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Immediate-type</td>
<td>Urticaria</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Hay fever</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bronchial asthma</td>
</tr>
<tr>
<td>II</td>
<td>Cytotoxic</td>
<td>HTR</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HDN</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ITP</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Some drug allergies</td>
</tr>
<tr>
<td>III</td>
<td>Immune complex</td>
<td>Immune glomerulonephritis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Serum sickness</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Arthus reaction</td>
</tr>
<tr>
<td>IV</td>
<td>Delayed-type</td>
<td>Tuberculin skin test</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Contact dermatitis</td>
</tr>
</tbody>
</table>

ITP = immune thrombocytopenic purpura; HDN = hemolytic disease of the newborn; HTR = hemolytic transfusion reactions.
D. Type IV reactions are delayed-type hypersensitivity reactions. CD4-positive T lymphocytes react with the foreign antigen and release lymphokines, some of which are chemottractants that attract PMNs, monocytes, and macrophages. These cells release substances such as proteases, collagenases, cathepsins, and TNF-α, which mediate inflammation. Examples include:
1. Tuberculin skin test
2. Contact dermatitis

VII. AUTOIMMUNITY is an expression of the Ir that occurs when the body’s self-tolerance system fails. The body’s immune cells are no longer able to recognize “self” and thus mount an Ir against its own antigens. This can result in a variety of apparently unrelated diseases known as autoimmune diseases. However, all autoimmune diseases involve immune complexes. The autoimmune response is strongly influenced by MHC antigens and can involve either Class I or Class II MHC proteins. Many autoimmune diseases are associated with specific Class II human leukocyte antigens (HLA), and determining an individual’s HLA type can help predict the risk of certain diseases.

A. Mechanisms. Autoantibodies form in response to many different immunogenic stimuli. The autoimmune response may be triggered by:
1. Sequestered antigens, which do not normally circulate in the blood and, as a result, fail to establish immunogenic tolerance
2. Foreign antigens, which may cross-react with self-antigens
3. Altered antigens, which become denatured or mutated because of physical, chemical, or biologic changes
4. Mutation of immunocompetent cells, which may become responsive to self-antigens
5. Dysfunction of T cells, which may lose their ability to regulate the Ir

B. Autoimmune diseases may be organ-specific or systemic (Table 5–7).
1. Organ-specific autoimmune diseases. Virtually any organ system can be affected by autoimmune diseases. Examples include:

| Table 5–7 Antibodies Associated with Autoimmune Diseases |
|----------------|--------------------------------|
| Disease         | Associated Antibodies |
| SLE             | Anti-DNA               |
|                 | Antinuclear             |
|                 | Antiribosome            |
|                 | Anti-DNP                |
| Primary biliary cirrhosis | Anti-acetylcholine receptor binding |
| Myasthenia gravis | Anti-acetylcholine receptor blocking |
| Pemphigus vulgaris | Anti-intrinsic factor |
| Sjogren’s syndrome | Anti-parietal cell |
| Multiple sclerosis | Antimyosin |
| Scleroderma      | Anticentromide          |
|                 | Anti-ScI                |
|                 | Anti-SS-A               |
|                 | Anti-SS-B               |
| Graves’ disease  | Antithyroglobulin       |
| Polymyositis     | Anti-Jo-1               |
|                 | Anti-Ku                 |
|                 | Anti-Mi-1               |
|                 | Anti-PM-1               |
| Goodpasture’s syndrome | Antiglomerular basement membrane |
| ITP             | Antiplasmin              |
| Insulin-dependent diabetes | Anti-islet cell |

DNP = deoxyribonucleoprotein; ITP = idiopathic thrombocytopenic purpura; SLE = systemic lupus erythematosus.
CHAPTER 5 Immunology and Serology

a. Hematologic disorders
   (1) Paroxysmal cold hemoglobinuria
   (2) Warm autoimmune hemolytic anemias (AIHAs)
   (3) Immune thrombocytopenia purpura (ITP)

b. Endocrine disorders
   (1) Graves’ diseases
   (2) Hashimoto’s thyroiditis
   (3) Insulin-dependent diabetes mellitus

c. Neuromuscular disorders
   (1) Myasthenia gravis
   (2) Multiple sclerosis

d. Renal disorders
   (1) Goodpasture’s syndrome
   (2) Tubulo-interstitial nephritis

e. Gastrointestinal disorders
   (1) Pernicious anemia
   (2) Primary biliary cirrhosis

2. Systemic diseases are characterized by widespread involvement. Autoantibodies are
   not confined to any one specific organ. Examples include:

a. Rheumatoid arthritis (RA)
   (1) General considerations, RA is characterized by the presence of abnormal
       circulating IgM autoantibodies known collectively as rheumatoid factor (RF).
       These antibodies react specifically with the Fc portion of IgG molecules. RF
       may also be seen in patients with chronic hepatitis, systemic lupus erythe-
       matosus (SLE), syphilis, hypogammaglobulinemia, or hypergammaglobuli-
       nemia.
   (2) Testing. There are a variety of tests for RF, including:
       (a) Latex fixation
       (b) Latex agglutination (commonly used test; considered positive for RF if the
           titer is at least 20)
       (c) Sheep red cell agglutination
       (d) Quantitation of IgM RF

b. Polymyositis
   (1) Description, Polymyositis is characterized by inflammation and degeneration
       of skeletal muscle.
   (2) Autoantibodies. Antinuclear antibodies (ANA), RF, and anti-Jo-1 antibodies
       are present.

c. SLE
   (1) Description, SLE is a systemic rheumatic disorder that is characterized by the
       presence of circulating immune complexes.
       (a) Epidemiology. It is most commonly seen in women and persons of African
descent.
       (b) Symptoms. More than half of SLE patients develop a characteristics rash
           or other skin abnormalities during the course of the disease. Other com-
           mon symptoms include myocarditis, lymphadenopathy, glomerulonephri-
           tis, and serositis.
       (c) Causes. SLE may be idiopathic or drug-induced. Drugs, such as pro-
           caineamide, phenytoin, methyldopa, penicillin, and sulfonamides, may
           cause a lupus-like syndrome. ANA are present and may persist for months
           after the drug has been discontinued.
   (2) Pathophysiology. Antibodies to native or altered self-antigens are produced
       and form circulating immune complexes with the antigens. Typically, the anti-
       gens involved are nuclear antigens, especially deoxyribonucleic acid (DNA).
       These complexes are deposited in various organs and cause inflammation,
       which eventually results in tissue injury.
   (3) ANA are autoantibodies that react with the body’s nuclear proteins, DNA, or
       histones. In general, they are neither organ-specific nor species-specific, and
they can cross-react with nuclear material from humans or animals. Although ANAs can be demonstrated in disease-free, healthy individuals, they are most often associated with various systemic rheumatoid diseases. The ANAs formed in SLE are usually IgG but may be IgM or IgA. Several different antibodies to DNA and DNA components may be seen in SLE. 

(a) Anti-ds (double-stranded)-DNA antibodies  
(b) Anti-ss (single-stranded)-DNA antibodies  
(c) Anti-ribonucleic acid (RNA) antibodies  
(d) Anti-extractable nuclear antigen antibodies  
(i) Smith (Sm) antigens  
(ii) Microsomal antigens (MA)  
(e) Anti-histone antibodies  
(f) Anti-deoxyribonucleoprotein (DNP) antibodies [LE factor; see Section VII B 2 c (d) (b)]

(4) Diagnosis (Table 5–8)

(a) ANA testing  
(i) The fluorescent antinuclear antibody test (FANA) is an indirect immunofluorescence technique used for ANA screening. Mouse or rat liver cells are commonly used as substrate cells. ANAs bind to the substrate cells’ nuclei. Fluorescein-conjugated antihuman globulin is added and binds to any antibodies present.  
(ii) ANA testing by immunohistochemistry is also commonly used. Human epithelial cells are used as substrate cells. Horseradish peroxidase (HRP)-conjugated antihuman globulin binds to any ANA that has bound to the nuclei of the cells.  
(iii) Interpretation. Positive results can be categorized by several different staining patterns: homogeneous, peripheral, speckled, nucleolar, and anticentromere.  
(b) The LE cell test demonstrates a phenomenon often seen in SLE patients as well as patients who have other diseases (e.g., RA, scleroderma, polymyositis). The LE factor is detected in more than 95% of patients with SLE, and it is responsible for the LE cell phenomenon. In the presence of the LE factor, disrupted homogeneous nuclei of lymphocytes and neutrophils are phagocytized by neutrophils. True LE cells must be distinguished from rosette formation (several neutrophils attempting to engulf one nucleus) and tart cells (monocytes phagocytizing whole cells or non-homogeneous nuclei).
VIII. IMMUNODEFICIENCIES. Deficiencies of the immune system include disorders of phagocytic cells, B lymphocytes, a combination of T and B lymphocytes, and the complement system.

A. Phagocytic cell deficiencies
   1. Phagocytic cell deficiencies result in a decreased ability to phagocytize and kill bacteria.
      a. Chronic granulomatous disease (CGD) is a genetic disease characterized by ineffective killing of bacteria by neutrophils.
         (1) Cause. CGD is caused by a defect in cytochrome b, which results in decreased hydrogen peroxide production. Hydrogen peroxide is necessary for producing the toxic superoxides that are critical in bacterial killing.
         (2) Diagnosis. The nitroblue tetrazolium (NBT) reductase test is used to detect impaired neutrophil phagocytosis. The neutrophils of CGD patients fail to reduce the NBT dye.
         (3) Symptoms. Patients with CGD suffer from recurrent infections caused by catalase-positive bacteria and yeast and fungi.
         (4) Treatment includes the use of GM-CSF or G-CSF and IFN-γ.
      b. MPO deficiency is inherited as an autosomal recessive trait and is one of the most common inherited disorders. The MPO in the primary granules of neutrophils is decreased or absent, and although phagocytosis takes place normally, bacterial killing is inefficient. Fungal killing is more seriously impaired than bacterial killing. Although otherwise healthy patients with MPO deficiency do not have an increased frequency of infection, diabetic patients who have this disorder may have an increase in Candida sp infections.
      c. Glucose-6-phosphate dehydrogenase (G6PD) deficiency is another inherited disorder in which the aerobic system of neutrophils is impaired. This deficiency results in a substantial decrease in the amount of hydrogen peroxide produced during phagocytosis, and thus decreased bacterial killing efficiency. Patients with G6PD deficiency experience recurrent bacterial infections.
      d. CR3 (iC3b receptor) deficiency is a rare, autosomal recessive trait characterized by a decrease or absence of specific complement component receptors on neutrophils, monocytes, and lymphocytes. These receptors are responsible for adherence-related functions. Abnormalities result in defective margination and diapedesis of neutrophils, impaired chemotaxis, and ineffective phagocytosis. T lymphocytes adhere poorly to target cells. Clinically, there is an increased frequency of bacterial infections, a decreased inflammatory response, and neutrophilia.
      e. Specific granule deficiency is inherited as an autosomal recessive trait. Neutrophils fail to develop specific granules during myelopoiesis, and as a result, patients who have this disorder experience severe, recurrent bacterial infections.
      f. Chédiak-Higashi syndrome is an inherited disorder that is characterized by the abnormal fusion of primary granules in neutrophils. During phagocytosis, degranulation is impaired, and little or no MPO is released into the phagosome. Patients who have Chédiak-Higashi syndrome have recurrent bacterial infections and are also characterized by albinism and extreme photosensitivity.
      g. Lazy leukocyte syndromes include:
         (1) Job syndrome, also known as hyperimmunoglobulin E, is characterized by poor chemotaxis and recurrent skin infections and abscesses.
         (2) Tuftsin deficiency. Tuftsin is a chemotaxin that also improves phagocyte motility, engulfment, and oxidative metabolism. Affected persons experience recurrent bacterial infections.
         (3) Actin dysfunction. A deficiency of the cytoskeletal protein actin can result in decreased cell motility and chemotaxis. Patients experience recurrent bacterial infections.

B. B-lymphocyte immunodeficiencies may be inherited or acquired and account for more than half of all immunodeficiencies. Affectedness varies widely, depending on the class
of immunoglobulin that is deficient. A deficiency of a minor immunoglobulin, such as IgD, causes little if any increase in the incidence of bacterial infections. However, because 75% to 85% of total immunoglobulin is IgG, an individual deficient in IgG would be significantly affected.

1. Bruton’s agammaglobulinemia is a sex-linked disorder that primarily affects men. It is usually recognized early in life when antibodies fail to develop. Pre-B cells may be found in the bone marrow, but they do not mature. Few mature B cells are found in the peripheral blood. Gamma globulin levels are markedly decreased. This disorder may be treated with gamma globulin preparations.

2. Common variable hypogammaglobulinemia is an acquired disorder in which one or two immunoglobulin classes are deficient. Total immunoglobulin levels may be normal, because a decrease in one immunoglobulin is often compensated by an increase in the production of another. Selective IgA deficiency is one of the most common of these deficiencies. Typically, only those patients whose disease includes IgG deficiency suffer from increased bacterial infections.

3. Neonatal hypogammaglobulinemia is caused by the normal immaturity of the neonate’s immune system. It corrects itself between the ages of 6 and 12 months as the infant’s immune system matures.

C. T-lymphocyte immunodeficiencies without an accompanying loss of B-cell function are rare, comprising only 7% of all immunodeficiencies. These disorders may be acquired or inherited.

1. DiGeorge syndrome results when the thymus gland develops abnormally during embryogenesis. Abnormalities of other endoderm-derived tissues are also seen. T lymphocytes are usually decreased, but may be normal. Most patients have a high CD4:CD8 ratio. Although antibody responses may be normal, cell-mediated immune responses are impaired.

2. Nezelof syndrome is an autosomal recessive disorder. Patients are athymic and are especially susceptible to viral and fungal infections, which can be fatal in these patients.

D. Combined B- and T-lymphocyte immunodeficiencies are the most serious of the immunodeficiencies, because both cell-mediated and humoral immune responses are affected.

1. Bare-lymphocyte syndromes are characterized by defects in Class I MHC antigen expression, Class II MHC antigen expression, or a combination of both. CD4-positive T lymphocytes are decreased in number, and B- and T-cell activation is reduced.

2. Severe combined immunodeficiency disease may be inherited as autosomal recessive or X-linked traits. All are characterized by markedly decreased numbers of both T and B lymphocytes.

3. Acquired immunodeficiency syndrome (AIDS) is caused by the human immunodeficiency virus 1 (HIV-1) or the human immunodeficiency virus 2 (HIV-2). The CD4-positive T lymphocytes are the primary target cells. Approximately 5% of B lymphocytes are also infected.

a. Transmission of the HIV virus is primarily through sexual contact with infected persons and parenteral routes (e.g., transfusion of infected blood and blood products). Other body fluids may also transmit the virus.

b. There are three stages of clinical manifestation of HIV infection.

(1) The primary stage may last from many months to years. Infected persons may experience an initial flulike illness, and then remain asymptomatic or exhibit only chronic lymphadenopathy for many years.

(2) The intermediate stage is also known as AIDS-related complex (ARC). There are physical symptoms but no opportunistic infections.

(3) The final stage, AIDS, is usually seen within 2 to 10 years after initial infection with the HIV virus. Opportunistic infections are common and include Pneumocystis carinii pneumonia, candidiasis, and histoplasmosis.

c. Effects on the immune system primarily involve CD4-positive T lymphocytes.

(1) The CD4:CD8 ratio is reduced from normal (2:1 to 0.5:1).

(2) CD4-positive T-cell counts are decreased.
(3) CD4-positive T-cell function is impaired.
(4) B-cell activation is abnormal.
(5) Macrophage function is impaired.
(6) NK cell function is impaired.

d. Laboratory detection of HIV infection. A number of tests have been developed to detect the presence of HIV antigens, antibodies, DNA, and RNA.

(1) Tests for antigens. The presence of HIV antigen indicates active infection.
(a) The HIV isolation technique can detect antigens before antibodies develop and can be used to monitor antiviral treatment. The patient’s monocytes are stimulated and grown in culture. The culture supernatant can be tested for the presence of antigens.
(b) A modified enzyme-linked immunosorbent assay (ELISA) can be performed on plasma, serum, cerebrospinal fluid (CSF), and culture fluids to detect the presence of HIV antigens.
(c) Immunofluorescence assay (IFA) can be used to detect HIV antigens.

(2) Tests for antibodies. The presence of HIV antibodies indicates prior exposure to the HIV antigen. Antibody development follows a predictable course following exposure. The p24 core protein is the major structural protein of the HIV-1 virus. IgM antibody against the p24 core protein usually develops within 6 to 8 weeks after infection. Within weeks, IgG antibodies against p24 appear, as do antibodies against envelope gene products (i.e., gp160, gp120, gp41) and polymerase gene products (i.e., p31, p51, p66).
(a) The ELISA is the most commonly performed screening test for HIV antibodies. Both false-positive and false-negative results may occur. Repeatedly reactive results necessitate confirmatory testing.
(b) Western blot assays are the most commonly used confirmatory test for HIV antibodies. Protein from disrupted virus is separated onto polyacrylamide gels by electrophoresis. False-positive reactions may occur.
(c) Other tests for HIV antibodies include:
(i) IFA
(ii) Radioimmunoassay (RIA)
(iii) Radioimmunoprecipitation assay
(iv) Slide agglutination tests

(3) Tests for DNA and RNA
(a) In situ hybridization and filter hybridization can be used to detect viral RNA. Cells from peripheral blood and lymph nodes can be used.
(b) Southern blot hybridization can be used to detect viral DNA in infected cells.
(c) Polymerase chain reaction (PCR) techniques can be used to amplify both RNA and DNA, which provides extremely sensitive systems for RNA and DNA detection.

IX. TECHNIQUES IN IMMUNOLOGY AND SEROLOGY

A. Agglutination assays demonstrate the presence of antigen-antibody reactions by the visible aggregation of antigen-antibody complexes. These tests are simple to perform and are often the most sensitive test method.

1. Flocculation tests. Antibody is detected when soluble antigen interacts with antibody and a precipitate is formed. Antigen is bound to reagent particles; visible agglutination results when the particles bind to antibody.

2. Latex agglutination. Antibody is bound to latex beads; visible agglutination occurs when antigen binds to the latex-bound antibody.

3. Direct bacterial agglutination. Antibodies bind to the surface antigens of bacteria in suspension, which results in visible agglutination.

4. Hemagglutination tests are used to detect antibodies to red blood cell (RBC) antigens. In passive or indirect hemagglutination tests, soluble antigens are adsorbed onto the
surface of RBCs. These antigens then bind to any corresponding antibody present, and the RBCs agglutinate.

5. Agglutination inhibition assays are very sensitive and can detect small amounts of antigen. In hemagglutination inhibition (HAI) assays, antigen and antibody are bound to RBCs. If antibody is present in the test sample, agglutination does not occur because there is already antibody bound to the antigen. Agglutination is a negative result. In other assays, antigen and antibody are bound to latex particles.

B. Precipitation assays. When both are present in proper proportions, antigens and antibodies interact and form visible precipitates. The largest amount of precipitation is seen when antigens and antibodies are present in optimal proportions; this is known as the equivalence zone. False-negative reactions can occur when either antigen or antibody is present in excess. (Web Color Images 5–12 and 5–13)

1. Double immunodiffusion (Ouchterlony method). Antigens and antibodies are allowed to diffuse in a semisolid medium such as agar or agarose. When the antigens and antibodies meet, a precipitate forms. Three basic reaction patterns can result from this interaction (Web Color Image 5–14).
   a. Identity. A single smooth arc of precipitation forms between the antigens and antibodies, which indicates that the antibodies are precipitating identical antigen specificities.
   b. Nonidentity. Two separate lines of precipitation cross each other, which indicates that the antigens and antibodies are unrelated and do not precipitate together.
   c. Partial identity. The two precipitating lines meet, forming a spur. This indicates that the antigens share some common epitopes, but that one of the antigens has a unique epitope.

2. Radial immunodiffusion (RID) is a single diffusion method that can be used to quantitate immunoglobulins and other serum proteins. Samples are introduced into wells cut in agarose containing antiserum and allowed to diffuse, usually overnight. The diameters of the resulting precipitation rings correspond to the amount of antigen present (Web Color Image 5–15).

3. Electroimmunodiffusion combines the speed of electrophoresis with the sensitivity of immunodiffusion.
   a. Countercurrent immunoelectrophoresis (CIE). This technique is essentially a double diffusion technique in which voltage is applied to move the antigens and antibodies together. A precipitin band forms when a zone of equivalence is reached.
   b. Rocket electrophoresis. This technique applies an electrical charge to a RID assay, which results in a rocket-shaped line of precipitation. The height of the rocket is proportional to the antigen concentration.

C. Electrophoresis

1. Immunoelectrophoresis (IEP) is often used to diagnose monoclonal gammopathies. After the serum or urine specimen is electrophoresed in a gel medium, a trough is cut in the agar parallel to the line of separated proteins. Monoclonal or polyclonal antisera are loaded into the trough, and the gels are incubated to allow the antigens and antibodies to diffuse toward each other. Precipitation lines become visible when a zone of equivalence is reached. Typically, control serum is run above the trough, and the patient sample is run below the trough for easy comparison (Web Color Image 5–16).

2. Immunofixation electrophoresis (IFE). After serum, urine, or CSF samples are electrophoresed in an agarose gel, cellulose acetate impregnated with antiserum is placed on the gel. The antiserum from the cellulose acetate diffuses into the gel, and antigen-antibody precipitates form. The cellulose acetate is stained to visualize the precipitation bands. IFE and IEP are often used together to work up monoclonal gammopathies.
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D. Labeling immunoassays can be qualitative or quantitative and can occur in the soluble or solid phase. Radioactive, enzyme, chemiluminescent, or fluorescent labels can be used.

1. RIA is a rapid and sensitive method that can be used to detect small amounts of antigen or antibody. However, exposure to radioisotopes can damage DNA and lead to radiation sickness, an increased incidence of neoplasms, or death. These potential hazards are a disadvantage to the RIA method (Web Color Image 5–17).

2. Radioallergosorbent test (RAST) is an RIA method specifically designed to measure antigen-specific IgE.

3. Radioimmunosorbent test (RIST) is a competitive binding technique used to quantitate IgE.

4. ELISA is similar in principle to RIA and has the same sensitivity, but uses an enzyme label instead of a radioactive label. HRP and alkaline phosphatase (ALP) are the most commonly used enzymes. ELISAs can be used to detect extremely small amounts of antigen or antibody (Web Color Image 5–18).

5. IFA are often used to identify antigens in tissue sections or air-dried smears of peripheral blood, bone marrow aspirates, touch preparations, or fine needle aspirate samples.

a. Labeling. Fluorescein isothiocyanate is the most commonly used fluorescent label. Labeling may be direct (using a labeled antibody) or indirect (using a labeled secondary antibody). After staining, the slides are washed and dried and immediately examined using a fluorescence microscope. Fluorescence quenches quickly when exposed to light, which is the major disadvantage of immunofluorescence methods (Web Color Image 5–19).

b. Immunocytochemical techniques are modifications of the direct and indirect immunofluorescence methods; enzyme labels such as HRP and ALP are used instead of fluorescent labels. These reaction products do not fade, so slides may be stored to provide permanent records.

E. Nephelometry (turbidimetry) photometrically measures the turbidity of solutions created by particles in suspension. It is accurate, rapid, and precise and is often used to quantify immunoglobulins, complement components, and immune complexes. This method can also be used to measure antigen concentration. The light source in nephelometry instruments produces a wavelength of 840 nm.

F. Neutralization tests. Neutralizing antibodies can destroy the infectivity of viruses, which provides the basis for assays that can determine the amount of viral antibody present. These techniques are often used to detect antibodies against herpes simplex virus Types 1 and 2 (HSV-1 and HSV-2) and echovirus.

G. Cellular assays. A wide variety of techniques exists for assessing the function of the cells of the immune system.

1. Functional assays. These tests are rarely performed except in large medical centers and reference laboratories. Some tests are even considered obsolete. Functional assays include the following.

a. ADCC assays use bacteria-infected tumor cells to assess the killing ability of NK cells.

b. The Boyden chamber uses chemotactic substances to assess the chemotactic response of neutrophils.

c. Cell-mediated monocytolysis uses tumor cells to assess the killing functions of monocytes.

d. Latex bead ingestion assesses phagocytic activity.

e. Lymphocyte transformation tests assess the ability of lymphocytes to respond to mitogens or specific antigens.

f. Lympholysis tests assess the ability of Tc to lyse labeled target cells.

g. Microcytotoxicity studies are used to detect HLA antigens and antibodies.
h. Migration inhibition techniques determine the ability of lymphocytes to produce chemotactic factors in response to granulocytes and monocytes.
i. Mixed-lymphocyte cultures are used to assess the human leukocyte antigen D (HLA-D) compatibility of donor and recipient lymphocytes.
j. NBT reductase tests are used to assess the intracellular killing ability of neutrophils.
k. Phagocytosis assays mix bacteria with neutrophils to assess the cells’ phagocytic ability.

2. Flow cytometry can be used to identify subpopulations of cells such as reticulocytes, granulocytes, T-cell subsets, B lymphocytes, and others. Fluorescent dyes, such as fluorescein, acridine orange, and phycoerythrin, are bound specifically to the cell marker of interest. Cells in suspension pass singly through a laminar-flow saline sheath. As the stained cells pass through a laser beam (usually argon or krypton), the dye is activated, and the cell fluoresces. The fluorescence is detected and collected by sensors placed at 90° relative to the source beam, and the information is processed by a computer (Web Color Image 5–20).

X. SYPHILIS SEROLOGY

A. Human syphilis is caused by the spirochete Treponema pallidum. Antibodies against treponemal antigens and nontreponemal cardiolipin antigens (Wassermann antigens) develop and elicit a cell-mediated and humoral Ir, which results in the formation of immune complexes.

B. Disease. Sexual contact with infected persons is the most common form of transmission of human syphilis. Transmission through blood or blood-product transfusion can occur but is rare now because of effective pretransfusion testing. In addition, syphilis can be passed from an infected pregnant woman to her fetus. There are four clinical stages of disease.

1. Primary (early) syphilis. Inflammatory lesions (chancres) appear 2 to 8 weeks after infection and last for 1 to 5 weeks. Serum tests for syphilis are positive in 90% of patients after 3 weeks. The antibodies that develop are predominantly IgM.

2. Secondary syphilis usually occurs 6 to 8 weeks after chancres first appear. This stage is characterized by a generalized rash, and secondary lesions may develop in the eyes, joints, or central nervous system (CNS). These lesions are highly contagious, but heal spontaneously within 2 to 6 weeks. Serologic tests are positive in secondary syphilis. Antibodies are mostly IgG.

3. The latent stage of syphilis is contagious and is generally considered to begin after the second year of infection. There are no clinical symptoms, although serologic tests are still positive. After 4 years, syphilis is not usually contagious; however, the disease may still be transmitted from mother to fetus.

4. Tertiary syphilis is characterized by granulomatous lesions known as gummata. These lesions may develop in skin, mucous membranes, joints, muscles, and bones, causing little or no clinical problems. Approximately 80% of patients experience CNS involvement, which can result in paralysis or dementia. Approximately 10% of patients develop cardiovascular problems, which can result in aortic aneurysm.

C. Congenital syphilis. Syphilis can be transmitted to a fetus after the 18th week of gestation. Treatment of the infected mother before the 18th week will prevent infection; treatment after the 18th week will cure it.

D. Treatment. Syphilis is easily and effectively treated. Penicillin is the drug of choice, although tetracycline or erythromycin can also be used. Treatment may or may not result in serologic tests becoming nonreactive, depending on the stage of the disease at the time of treatment.
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1. Primary stage. A seropositive patient in the primary stage of disease usually becomes nonreactive approximately 6 months after treatment.

2. Secondary stage. If treatment occurs during the secondary stage, the patient usually becomes nonreactive within 12 to 18 months after treatment. Patients treated 10 years or more after infection may always remain seropositive.

E. Tests for syphilis are based on the detection of nontreponemal antibodies or treponemal antibodies.

1. Nontreponemal antibody detection. Reagin antibodies are formed after exposure to *Treponema pallidum* and react with lipoidal antigens used in screening tests for syphilis. The most commonly performed reagin tests are:
   a. The Venereal Disease Research Laboratory (VDRL) slide test. The VDRL is a qualitative agglutination test using heat-inactivated patient serum. CSF can also be used. The test can be modified and used as a quantitative test.
   b. The rapid plasma reagin (RPR) test. The RPR is an agglutination test. In addition to lipoidal antigens and cholesterol, the antigen reagent contains charcoal to facilitate macroscopic interpretation of results. Unheated serum is the specimen of choice, although plasma may be used.
   c. Results. VDRL and RPR test reactions are graded as nonreactive (NR), weakly reactive (WR), or reactive (R). False-positive and false-negative reactions may occur.
      (1) False-positive reactions may be caused by:
         (a) SLE
         (b) RA
         (c) Infectious mononucleosis
         (d) Pregnancy
         (e) Old age
      (2) False-negative reactions may be caused by:
         (a) Technical errors
         (b) Low antibody titers
         (e) Prozone phenomenon

2. Treponemal antibody detection. The serum of patients who have syphilis also contains an antibody distinct from the antireagin antibody. Tests for these antibodies are used as confirmatory tests when reactive results are obtained using screening methods. The most commonly used treponemal tests are:
   a. The fluorescent treponemal antibody absorption test (FTA-ABS). The FTA-ABS test detects treponemal antibodies by using a killed suspension of *T. pallidum* as an antigen and a fluorescein-conjugated antihuman globulin reagent.
   b. The microhemagglutination assay for *T. pallidum* (MHA-TP) uses RBCs coated with treponemal antigens to detect antibodies.

XI. ACUTE PHASE PROTEINS are a group of plasma proteins whose levels increase significantly and independently during the acute phase of an inflammatory process. They are primarily produced in the liver by the parenchymal cells.

A. α1-antitrypsin is a serine protease inhibitor that inhibits the action of specific proteases that cause lung damage and pulmonary inflammation. Heterozygous deficiencies result in increased risk of liver disease, glomerulonephritis, and connective tissue diseases. Homozygous deficiencies result in premature emphysema and liver disease.

B. α2-macroglobulin is a protease inhibitor that plays a role in the coagulation, fibrinolytic, and complement components of hemostasis.

C. Ceruloplasmin is a major copper-transport protein. An absence or deficiency of ceruloplasmin is associated with Wilson’s disease.
D. C-reactive protein (CRP) is normally present in trace amounts in serum, but may increase to 1000 times normal in many inflammatory processes. It is the first of the acute reactive proteins to appear following tissue injury or inflammation. CRP levels can be used to assess disease severity and monitor therapy. Elevated levels are seen in rheumatic diseases such as RA, bacterial and viral infections, burn injuries, malignancies, tuberculosis, and renal transplantation. Levels also rise rapidly following myocardial infarction and correspond with elevated CK-MB levels. The latex agglutination test is the most commonly used method for CRP testing.

E. Fibrinogen plays an active role in wound healing after tissue injury. It is primarily responsible for an elevated erythrocyte sedimentation rate (ESR).

F. Haptoglobin is a plasma protein that binds to free hemoglobin. Decreased levels may be caused by intravascular hemolysis or decreased synthesis secondary to liver disease. Haptoglobin levels increase twofold to fourfold following tissue injury.

XII. HEPATITIS is an inflammation of the liver and usually refers to the diseases caused by a group of viruses identified as hepatitis A, B, C, D, and E.

A. Hepatitis A (infectious hepatitis) is caused by the hepatitis A virus (HAV).
   1. Transmission. HAV is transmitted most commonly through the fecal-oral route and is frequently seen in epidemics in areas with poor sanitation. Raw shellfish from contaminated water can also transmit the disease.
   2. Disease course. HAV infections are almost always acute and self-limiting. There is no carrier state. Symptoms, if present, are vague and relatively nonspecific; patients may complain of fatigue, malaise, and anorexia. Jaundice may be present, although most patients are anicteric. Many cases are subclinical, especially those in children.
   3. Laboratory diagnosis. Liver function test results, especially alanine aminotransferase (ALT), are elevated. Total bilirubin levels may be elevated. Antibodies to HAV can be detected by enzyme immunoassay (EIA) and RIA methods. IgM anti-HAV antibodies develop during the acute phase of the disease and persist for 3 to 12 months after onset of the disease. As IgM titers decrease, levels of IgG anti-HAV antibodies increase and persist throughout life. These IgG antibodies confer lifelong immunity.
   4. Prevention. Household and sexual contacts of infected persons should receive immune globulin injections within 2 weeks of exposure. A recently developed vaccine against HAV is now available.

B. Hepatitis B virus (HBV) was formerly known as the Australia or hepatitis-associated antigen. Box 5–1 summarizes the order of appearance of HBV markers.
   1. Transmission. HBV is transmitted parenterally or through sexual contact with infected persons. Common parenteral routes include intravenous drug use, transfusion of contaminated blood or blood products, and cutaneous or mucous membrane exposure (e.g., needlestick injuries, splashes in the eyes, nose, or mouth).
   2. Disease course. The average incubation period is 2 to 3 months. HBV infections may be acute, chronic, or fulminant, or the patient may be a chronic asymptomatic carrier. Symptoms are similar to those seen in HAV infections. Jaundice may or may not be present. Approximately 95% of all cases of hepatitis B are acute. Acute HBV

Box 5-1 Order of Appearance of Hepatitis B Markers

- Hepatitis B surface antigen (HBsAg)
- Hepatitis B e antigen (HBeAg)
- Hepatitis B core antibody (HBcAb)
- Hepatitis B e antibody (HBeAb)
- Hepatitis B surface antibody (HBsAb)
infections can be complicated by circulating HBV antigen-antibody complexes, which can cause polyarteritis, arthritis, glomerulonephritis, pancreatitis, or cryoglobulinemia. Chronic hepatitis B can progress to cirrhosis, which may later progress to hepatocellular carcinoma.

3. Laboratory diagnosis. Elevated ALT levels peak approximately the same time symptoms appear. Serologic tests not only identify HBV as the cause of the disease, but can also be used to pinpoint the stage of the disease.

a. Hepatitis B surface antigens (HBsAg) appear 1 week to 2 months after exposure, and approximately 2 weeks to 2 months before the onset of symptoms. HBsAg disappears during the convalescent phase of acute disease and is an indicator of acute infection or chronic infection with unresolved antigenemia.

b. Hepatitis B surface antibodies (HBsAb) appear as the antigens disappear. The presence of these antibodies indicates recovery and lifelong immunity.

c. Hepatitis B core antibodies (HBcAb) appear shortly after the surface antigens appear. At this time, the ALT levels begin to rise. HBcAb persist throughout life and are a marker for previous infection.

d. Hepatitis Be antigen appears before the onset of clinical disease, after the appearance of the surface antigens, and disappears within approximately 2 weeks. The presence of the Be antigen indicates active viral replication, and HBV is most infectious when the Be antigen is detectable.

e. Antibodies to hepatitis Be antigen appear shortly after the antigen disappears. Their presence in patients with acute hepatitis B suggests that the infection is resolving. In most cases of chronic hepatitis B infection, the presence of hepatitis Be antibodies indicates that the infection is resolving or that there is no complicating liver disease. Hepatitis Be antibodies may be seen in chronic asymptomatic carriers.

4. Prevention. Avoidance of high-risk behavior (e.g., intravenous drug abuse and sexual contact with infected persons) is a major factor in preventing HBV infection. A vaccine against HBV has been available since 1982. In a health care setting, HBV vaccination and the use of universal precautions can greatly reduce the risk of occupationally acquired HBV. The HBV vaccine also protects against HDV infection, because HDV infection only occurs with HBV infection.

C. Hepatitis C virus (HCV) has recently been identified as the causative agent in the majority of cases of what was previously known as non-A, non-B hepatitis (see Web Case Study 5–1).

1. Transmission. Like HBV, HCV is transmitted most commonly by parenteral routes or through sexual contact with infected persons. The majority of post-transfusion non-A, non-B hepatitis cases are caused by HCV.

2. Disease course. The average incubation period is 7 to 8 weeks. HCV infections may be acute or chronic. Symptoms are similar to those seen in HAV and HBV infections. Approximately 50% of HCV-infected patients are chronic carriers. Approximately 20% of these patients develop cirrhosis, and approximately 20% of those patients eventually develop hepatocellular carcinoma. HCV is also associated with immune-complex glomerulonephritis.

3. Laboratory diagnosis. Elevated ALT levels are associated with HCV infection. EIA and RIA assays have been developed to detect antibodies to the HCV antigens, but a high number of false-positive results are seen with these methods. Identifying infected persons is also difficult because antibody tests are positive in only 70% to 85% of patients with post-transfusion HCV and in only 50% of patients with disease from other causes. Additionally, it can take up to 12 months for an infected person to seroconvert and test positive for HCV antibodies. Serum or liver tissue can be analyzed for HCV RNA by PCR, but this method largely remains a research tool.

4. Prevention. As with HBV, avoiding high-risk behavior (e.g., intravenous drug abuse and sexual contact with infected persons) is important in preventing HCV infections. No vaccine for HCV currently exists.
D. **Hepatitis D** is associated exclusively with HBV infections, either as a co-infection or as a superinfection. It is seen most commonly in intravenous drug users and hemophiliacs. No vaccine is available. The only means of preventing hepatitis D infection is to prevent HBV infection.

E. **Hepatitis E** causes sporadic and epidemic hepatitis in developing countries such as India, Pakistan, Africa, and Mexico. The disease clinically resembles HAV infections and is transmitted through the fecal-oral route. There is no chronic infection, and the hepatitis E virus is not associated with hepatocellular carcinoma.

### XIII. STREPTOCOCCAL SEROLOGY

**Streptococcus pyogenes** is a gram-positive coccus responsible for a number of human infections, some of which can have serious sequelae. The M protein is the major virulence factor for *S. pyogenes*; more than 60 M serotypes have been identified.

A. **Bacterial toxins.** Two hemolysins are produced by virtually all strains of *S. pyogenes*.

1. **Streptolysin O (SLO)** is an oxygen-labile enzyme that causes hemolysis by binding to cholesterol in the RBC membrane. It is antigenic, and the presence of antibodies to SLO is an indicator of recent streptococcal infection.

2. **Streptolysin S** is a nonantigenic, oxygen-stable enzyme. It causes hemolysis by disrupting the selective permeability of the RBC membrane.

B. **Infections and sequelae**

1. **Skin infections** caused by *S. pyogenes* include:
   a. Cellulitis
   b. Impetigo
   c. Erysipelas

2. **Upper respiratory tract infections** caused by *S. pyogenes* are characterized by fever, sore throat, and pharyngeal edema.

3. **Scarlet fever** is caused by a strain of *S. pyogenes* that produces an erythrogenic toxin, which results in a characteristic rash. Fever and sore throat are also present.

4. **Rheumatic fever (RF)** is a complication seen following upper respiratory tract infections. All M serotypes that cause pharyngitis have been implicated in RF. RF results in damage to heart valves, and patients with rheumatic heart disease have an increased risk of developing endocarditis and other cardiac problems in later years.

5. **Post-streptococcal glomerulonephritis** may occur after pharyngitis or skin infections. Only a few M serotypes cause this type of glomerulonephritis. Patients have an increased risk of developing renal failure later.

6. **The mechanisms** by which these sequelae occur are not fully understood. Antibodies to streptococcal cell membranes cross-react with myosin in cardiac muscle cells, which results in cell damage. Antigen-antibody complexes form at the glomerular basement membrane and attract inflammatory cells that cause renal tissue damage.

C. **Laboratory diagnosis.** During an infection with *S. pyogenes*, the SLO produced elicits an Ir, and specific antibodies are formed. These antibodies neutralize the hemolytic activity of the SLO; this neutralization provides the basis for the most commonly used test in the detection of streptococcal infections.

1. **Antistreptolysin O (ASO) titer.** The ASO titer begins to increase approximately 7 days after infection and peaks after 4 to 6 weeks.
   a. **Principle.** SLO is added to serial dilutions of patient serum, along with group O RBCs as indicator cells. If the patient serum contains antibodies against SLO, the antibodies will complex with the corresponding antigens. These complexes block the hemolytic activity of the antigen, and no hemolysis occurs. The ASO titer is reported as the reciprocal of the highest dilution that shows no hemolysis and is expressed in Todd units.
b. Normal values. ASO values vary widely among healthy individuals, making it difficult to establish normal values. Most healthy adults have ASO titers of less than 166 Todd units, with the usual titer decreasing after 50 years of age. Although an elevated titer is generally regarded as evidence of recent streptococcal infection, a 30% rise in titer above a previous level is of greater significance than a single titer. The titer can remain elevated for weeks or months following acute disease. Approximately 80% to 85% of patients with RF have increased ASO titers.

2. Anti-DNase B (AD-B). Streptococci produce the enzyme deoxyribonuclease B (DNAse B). The anti-DN-B test is a neutralization test that can demonstrate recent streptococcal infection. Anti-DN-B neutralizes the activity of DNAse B. Anti-DN-B levels are increased in the 15% to 20% of RF patients who do not have elevated ASO titers.

3. Other tests. Several rapid tests are now available to detect streptococcal antigens. The advantage is their speed over other test methods. However, a significant number of false-negative results occur.

XIV. EPSTEIN-BARR VIRUS (EBV) SEROLOGY

A. Description. EBV is the causative agent of Burkitt’s lymphoma, nasopharyngeal carcinoma, and most commonly, infectious mononucleosis (IM). The virus is ubiquitous; 80% to 90% of healthy adults have EBV antibodies. EBV infects B lymphocytes.

B. Infectious mononucleosis (IM) is an acute, self-limiting disease typically seen in young adults. The disease is characterized by fever, sore throat, cervical lymphadenopathy, splenomegaly, and mild hepatitis. The WBC count is elevated, and reactive lymphocytes are seen in the peripheral blood. There is a relative and absolute lymphocytosis. The average incubation period is approximately 2 to 8 weeks (see Web Case Study 5–2).

C. Antigens and antibodies

1. Viral capsid antigen (VCA) is found in the cytoplasm of EBV-infected lymphocytes. IgM antibodies against VCA are detectable early in the infections, but disappear within 2 to 4 months. IgG antibodies against VCA develop within 1 week after infection and can persist for life.

2. Early antigen-diffuse (EA-D) and early antigen-restricted (EA-R) antigens are found in the cytoplasm of infected B lymphocytes. EA-D is also found in the nucleus. IgG antibodies to EA-D can be indicators of active disease. IgG antibodies to EA-R are sometimes seen in young children who have active IM infection, but not in infected young adults.

3. Epstein-Barr nuclear antigen (EBNA) is found in the nuclei of all infected cells. IgG antibodies to EBNA develop slowly but can remain detectable throughout life.

4. Heterophile antibodies are stimulated by one antigen and will react with unrelated antigens from different mammalian species. The heterophile antibodies of IM are IgM antibodies and are seen in 50% to 70% of patients with IM. They persist for 4 to 8 weeks after infection.

D. Testing

1. The Monospot test is based on the principle that horse RBCs are agglutinated by the heterophile antibodies of IM. Horse RBCs contain both Forssman and IM antigens; patient serum must be differentially absorbed to distinguish the antibodies. The patient serum is absorbed with guinea pig kidney, which will absorb only heterophile antibodies of the Forssman type, and beef erythrocyte stroma, which will absorb only the heterophile antibodies of IM. The Monospot test is positive if the horse RBCs are agglutinated by the patient serum absorbed with guinea pig kidney, and not agglutinated by the patient serum absorbed with beef erythrocyte stroma.

2. The Davidsohn differential test can distinguish heterophile sheep cell agglutinins in human serum caused by IM, serum sickness, and Forssman antigen.
3. The Paul-Bunnell test can detect only the presence or absence of heterophile antibodies. It cannot determine the specificity of the antibodies.

XV. RUBELLA SEROLOGY

A. The rubella virus is the causative agent of acquired rubella, which is also known as German measles or 3-day measles. It is highly contagious, although its incidence has decreased dramatically because of vaccination. Rubella is characterized by rash, fever, and lymphadenopathy. Both IgG and IgM antibodies develop during acute infection. IgM antibodies disappear within a few weeks, but IgG antibodies persist and confer lifelong immunity.

B. Congenital rubella syndrome is caused by infection during pregnancy and can result in a wide spectrum of birth defects. In utero infection can also result in fetal death. The fetus is most likely to develop anomalies if the mother becomes infected during the first month of pregnancy.

C. Testing. Because IgM antibodies disappear quickly after infection, their presence is indicative of a current or very recent infection. However, because IgG antibodies appear early in the course of the disease and persist, their presence does not necessarily indicate current or recent infection. Because IgM does not cross the placental barrier, demonstration of IgM rubella antibodies in a neonate is diagnostic of congenital rubella syndrome. Pregnant women are often tested for rubella early in their pregnancy. Several methods of detecting rubella antibodies are commonly used:

1. HAI is the most widely used test for the detection and quantitation of rubella antibodies. It does not distinguish between IgG and IgM antibodies and is therefore most useful as an indicator of immune status. A titer of at least 8 is considered to be indicative of prior infection.

2. Other tests include:
   a. Passive latex agglutination, which is faster and more convenient than the HAI
   b. Quantitative methods, such as EIA and IFA

XVI. FEBRILE DISEASE SEROLOGY

A. Febrile diseases are a group of microbial infections characterized by fever and the production of antibodies known as febrile agglutinins. These diseases include:

1. Brucellosis, which is caused by the bacteria *Brucella abortus*
2. Paratyphoid fever, which is caused by *Salmonella paratyphi*
3. Rocky Mountain spotted fever, which is caused by rickettsiae
4. Tularemia, which is caused by *Francisella tularensis*
5. Q fever, which is caused by rickettsiae

B. Tests for febrile diseases include:

1. Widal's test, which can detect antibodies in typhoid fever, tularemia, and brucellosis
2. The Weil-Felix test, which is an agglutination test based on the cross-reactivity of rickettsial antibodies with antibodies to the somatic “O” antigen of the OX-19 and OX-2 strains of *Proteus vulgaris* and the OX-K strain of *Proteus mirabilis*

   a. The Weil-Felix test is useful for identifying several rickettsial diseases, such as murine typhus and Q fever.
   b. Titers of 160 are usually considered significant.

XVII. BORRELIA BURDORFERI SEROLOGY

A. Organism—Borrelia Burgdorferi is a bacterium discovered by Dr. Willy Burgdorfer in 1982 who isolated the spirochetes from mid-guts of Ixodes ticks.
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B. Transmission of Lyme Disease is spread by the bite of infected ticks of the genus *Ixodes*. The preferred host for both the larval and nymphal life cycle of tick is the **white footed mouse**. White-tailed dear is the preferred for the adult stage. Lyme disease does occur in domestic animals including dogs, horse and cattle.

C. Stages of Lyme disease include the following:
   1. **Stage one: Early infection**
      Distinct expanding red rash ("bull’s eye" or target rash appearance): erythema chronicum migrans appears in 60% to 80% of infected individuals 2 to 32 days after being bitten by infected tick. Spirochetes can be isolated from the leading edge of the classic bull’s eye rash (erythema chronicum migrans) which usually persists up to 1 month.
   2. **Stage two: Dissemination stage**
      Usually occurs days to weeks after initial infection. Spirochetes spread hematogenously to body tissues by way of bloodstream and disease may take various forms. Symptoms include muscle and joint pain, fatigue, chills and fever, headache, swollen lymph nodes, secondary annular skin lesions.
   3. **Stage three: Chronic disseminated**
      Commonly involves intermittent episodes of joint pain which is often misdiagnosed as arthritis. Some common clinical manifestations of this stage can include meningitis, Bell’s palsy, cardiac involvement, migratory pain to joints, tendons, muscle and bone.

D. **Diagnosis**
   1. **Patient History.** Lyme disease is difficult to diagnose because symptoms and signs mimic those of so many other diseases. Diagnosis should include a close evaluation of patient history to exposure with ticks.
   2. **Serologic tests.** Diagnosis can be made if a fourfold increase in titer is detected between initial infection and 6 to 8 weeks later.
      a. **IFA** is the most common method to diagnose Lyme disease. IFA searches for antibody against the antigen, which is attached with a fluorescent tag. False positive due to rheumatic disease (different pattern than Lyme disease)
      b. **ELISA,** which is more sensitive than IFA. The color intensity is proportional to amount of Ab present. False positives can be caused by other spirochetal disease such as syphilis.
      c. **DNA probe via western blotting** which is very sensitive as well as an expensive technique; not good for screening.
      d. **Culture of Borrelia Burgdorferi.** This organism can be cultured in the laboratory via Barbour-Stoenner-Kelly (BSK) medium at 33°C.
      e. **PCR**
      f. **Gundersen lyme test (GLT)** (also known as the borreliacidal-antibody test BAT) uses flow cytometry to detect borreliacidal antibodies which helps eliminates cross-reactivity and increases sensitivity.

E. **Treatment**
   Antibiotics. Drug of choice is doxycycline (semisynthetic derivative of tetracycline), which one takes a minimum of 4 to 6 weeks.

XVIII. TRANSPLANT IMMUNOLOGY

A. Types of grafts
   1. Autograft. Self-tissue transferred from one body site to another in the same individual
   2. Isograft (syngraft). Tissue transferred between genetically identical individuals
   3. Allograft (homograft). Tissue transferred between genetically different members of the same species
   4. Xenograft (heterograft). Tissue transferred between different species.

B. Graft acceptance and types of rejections
   1. Graft acceptance is when revascularization and healing lead to a repaired site in about 2 weeks.
2. Two types of graft rejections
   a. **First-set rejection** - First time a graft is encountered and rejected. The immune system attacks and ultimately destroys the “nonself tissue,” which occurs 10 to 14 days after transplantation.
   b. **Second-set rejection** - The second time the same set of “nonself tissue” is encountered which is usually rejected within 6 days.

3. Mechanisms involved in graft rejection
   a. **Graft rejection** is caused by cell-mediated immune response to alloantigens expressed on cells of the graft. Both delayed-type hypersensitivity and cell-mediated cytotoxicity reactions have been implicated.
   b. **Two stages of rejection**
      (1) **Sensitization stage (Stage 1)**, where CD4 and CD8 T cells recognize alloantigens expressed on cells of the foreign graft and proliferate in response. The host T helper cell becomes activated when it interacts with an antigen presenting cell. Depending upon the tissue, different populations of cells within a graft may function as antigen presenting cells. Migration of passenger leukocytes from a donor graft to regional lymph nodes of the recipient results in the activation of T-H cells in response to different Class II MHC antigens expressed by the passenger leukocytes. These activated T-H cells then induce generation of cytotoxic T cells of which mediate graft rejection.
      (2) **Effector stage (Stage 2)**. Most are cell-mediated reactions involving delayed-type hypersensitivity and CTL-mediated cytotoxicity; less common mechanisms are antibody-plus-complement lysis and destruction by antibody-dependent cell-mediated cytotoxicity (ADCC)
   c. **Types of rejection**
      (1) **Hyperacute rejection reactions** occur within the first 24 hours after transplantation due to pre-existing antibodies specific for allogenic MHC antigen. Due to pre-existing antibodies specific for allogenic MHC antigen. This can be caused by repeated blood transfusions, repeated pregnancies, or previous graft.
      (2) **Acute rejection** reactions usually begin in the first few weeks after transplantation.
      (3) **Chronic rejection** reactions can occur from months to years after transplantation and usually develops months or years after acute rejection reactions have subsided. Mechanisms of chronic rejection include both humoral and cell-mediated response by the recipient

C. **Donor testing and tissue typing**. Tests performed on tissues donors usually are ABO/Rh, CMV, RPR and FTA, HbsAg, EBV, anti-HIV 1 and 2, HbcAg, HTLV I&II, parvovirus B19, HCV, and HLA matching. The donor and recipient must be typed and matched for ABO and HLA antigens. HLA-identical donors have the lowest rate of rejection.

D. **Immunosuppression**. All allogeneic transplantation requires some degree of immunosuppression if the transplant is to survive. The disadvantage is that most are nonspecific, making the individual more immunocompromised and, therefore, more susceptible to infection. Many immunosuppressive measures are aimed at slowing the proliferation of activated lymphocytes. Any rapidly dividing nonimmune cells are also affected, therefore serious complications can occur including increased risk of cancer, hypertension, and metabolic bone disease.
   1. **Azathioprine (Imuran)**, a potent mitotic inhibitor
   2. **Cyclophosphamide** disrupts the DNA chain
   3. **Methotrexate** acts as a folic acid antagonist to block purine biosynthesis
   4. **Corticosteroids** are antiinflammatory drugs that affect activated macrophages and decrease the expression of HLA antigens
   5. **Cyclosporine** which reduces the expression of IL-2 receptors on affected lymphocytes and decreases lymphokine production of T helper cells. Prevents T-cell activation
   6. **FK-506 (Tacrolimus)**, T cell inhibitor; less toxic to kidneys, liver and CNS
Table 5–10 Tumor Markers

<table>
<thead>
<tr>
<th>Tumor Marker</th>
<th>Tumor Association</th>
</tr>
</thead>
<tbody>
<tr>
<td>CEA (Carcinoembryonic antigen)</td>
<td>Elevated in patients with colon cancer, colectis, pancreatitis, and prostate cancer.</td>
</tr>
<tr>
<td>AFP (Alpha1-fetoprotein)</td>
<td>Elevated in the serum of patients with liver tumors, and germ cell tumors such as testicular cancer, and can be used as a marker of disease status.</td>
</tr>
<tr>
<td>ß-hCG (Beta Subunit of Human Chorionic Gonadotropin)</td>
<td>In men, ß-hCG can be used as a serum marker to monitor treatment and recurrence of testicular cancer. In women, increased in malignancies.</td>
</tr>
<tr>
<td>CA-125</td>
<td>Expressed in most nonmucinous epithelial ovarian tumors. Benign, borderline, and malignant ovarian neoplasms usually express this Ag. Also detected in carcinomas arising from the fallopian tube, endometrium, endocervix, pancreas, liver, colon, breast, and lung</td>
</tr>
<tr>
<td>CA15-3</td>
<td>Used in the detection of breast cancer, also found in endocarcinomas of the ovaries, lungs, and pancreas; greatest increase in metastatic disease rather than in primary breast cancer. May also be elevated in benign disease such as chronic hepatitis, cirrhosis of the liver, sarcoidosis, tuberculosis, and SLE</td>
</tr>
<tr>
<td>CA19-9</td>
<td>Aids in diagnosing colon, gastric, hepatobiliary, and pancreatic cancer. Decreases after surgical removal of tumor</td>
</tr>
<tr>
<td>PSA (Prostate-Specific Antigen)</td>
<td>Prostate tissue specific; detects cancer of the prostate, increased levels are detected in benign or malignant tumor inflammation.</td>
</tr>
</tbody>
</table>
3. **Malignant**: Immortal, progressively invasive, CANCER, invades normal tissues

4. **Carcinoma**: Tumors that arise from endodermal or ectodermal tissue

5. **Leukemia and lymphoma**: Malignant tumors of hematopoietic cells of the bone marrow

6. **Sarcoma**: Derived from mesodermal connective tissues

7. **Metastasis**: Moves through the blood or lymph to new sites; small clumps of cells that break off and spread where they continue to grow

8. **Proto-oncogenes**: Genes that normally regulate the initiation and execution of normal cell division

9. **Oncogenes**: The mutant forms of proto-oncogenes that contribute to various tumor types or malignant transformation (Table 5–9)

**B. Tumor antigens**

**Tumor-specific antigens**: are not found on normal somatic cells but result from mutations of genes.

**Tumor-associated antigens**: are not unique to tumor cells but antigen expression has been altered and can be expressed in excess were it would not normally exist.

a. oncofetal antigens- are expressed on tumors and on normal fetal cells. Examples include AFP and CEA. See Table 5–10.

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### Online Menu for This Chapter

Go to thePoint.lww.com/Hubbard2e for the following:

- Web Color Images 5–1 through 5–20
- Case Studies 5–1 and 5–2
- Study Questions and Answers
I. INTRODUCTION

A. Definitions

1. **Antigens** (Ag) are substances that are recognized as foreign by the body and, therefore, elicit immune responses. **Blood group antigens** exist on the surface of red blood cells (RBCs). Only a relatively small number of blood group antigens are considered clinically significant, but more than 600 have been described.

2. **Antibodies** (Ab) are immunoglobulins (Ig) that are developed in response to the presence of antigens.
   a. **Heteroantibodies** (xenoantibodies) are antibodies produced in response to antigens from another species.
   b. **Alloantibodies** are formed in response to antigens from individuals of the same species and are the type of antibodies involved in transfusion reactions.
   c. **Autoantibodies** are made in response to the body’s own antigens.

B. **Immunoglobulins** are produced and secreted by activated B lymphocytes. All share the same basic structure of two heavy chains and two light chains held together by disulfide bonds. There are **five classes**—IgG, IgA, IgM, IgE, and IgD. Only IgG, IgM, and (rarely) IgA antibodies are produced against RBC antigens. (See Web Color Image 6–1.)

   1. IgA antibodies against RBC antigens usually occur with IgG and IgM antibodies having the same specificity. They do not cross the placental barrier and do not fix complement. IgA antibodies can cause agglutination in saline.
   2. IgG antibodies account for the majority of the clinically significant antibodies directed against blood group antigens. There are **four subclasses** of IgG: IgG1, IgG2, IgG3, and IgG4.
      a. Most IgG antibodies contain all four subclasses, but some are predominantly or exclusively composed of a single subclass.
      b. The subclasses display different biologic properties.
         (1) All bind to the crystallizable fragment (Fc) receptors on macrophages; all can cross the placental barrier; and all but IgG4 are able to bind complement through the classic pathway.
         (2) IgG1 and IgG3 bind complement much more efficiently than does IgG2.
         (3) IgG1 comprises 65% to 70% of the total IgG found in serum.
   3. IgM antibodies agglutinate very strongly in saline, so they are considered **complete** antibodies. IgM exists in serum as a **pentamer** and cannot cross the placental barrier. It strongly fixes complement through the classic pathway.
C. Antigen-antibody reactions. Antigen-antibody reactions important to immunohematology involve the agglutination of erythrocytes by antibodies.

1. Hemagglutination is agglutination of the RBCs. It occurs in two stages.
   a. Sensitization occurs when the antigen-binding sites of the antibodies become closely associated with the antigenic determinants of the RBC membrane. The antibodies and binding sites are held together loosely by noncovalent bonds.
   b. Visible agglutination occurs when several RBCs are physically joined together by the antigen-antibody union. This stage depends on many factors, such as the pH, temperature, and ionic strength of the suspension medium.
      (1) The use of proteolytic enzymes such as papain or ficin can enhance cell-to-cell contact.
      (2) RBCs sensitized by incomplete antibodies (antibodies that will not react in saline) agglutinate when antiserum against human IgG is added.

2. The amount of available antigen and antibody also affects hemagglutination.
   a. Prozone occurs when antibody molecules are in excess of available antigenic sites, resulting in false-negative reactions.
   b. Equivalence—optimal proportions of antigen and antibody present—allows hemagglutination to occur.
   c. Antigen excess (postzone) also results in false-negative reactions.

D. Complement proteins play a number of biologic roles, but the most important role in immunohematology is their ability to lyse the cell membranes of antibody-coated RBCs. Because complement components are unstable and heat liable, it is important for serum specimens to be fresh for blood bank testing. The native precursor components are numbered from 1 to 9 with subcomponents of the proteins receiving the letters from a to e as they are cleaved. Complement is activated through two pathways: the classic pathway and the alternate pathway. Although these two pathways are independent, they converge at the C5 reaction, and the reactions from C5 to C9 are common to both pathways. (See Web Color Image 6–2.)

1. The classic pathway is activated by both IgG and IgM antibodies when the C1 component binds to the Fc portion of the antibody molecule. IgM and IgG3 antibodies are most efficient at complement activation.
2. The alternate pathway does not require specific antibody for activation, but is instead triggered by polysaccharides and lipopolysaccharides on the surfaces of certain target cells.
3. The reactions that take place from C5 to C9 are termed the membrane attack complex and result in lesions on the RBC surface. These lesions allow the rapid passage of ions, and the cell lyses from osmotic pressure changes.

II. BLOOD GROUP SYSTEMS

A. The ABO blood group system is the most important system in transfusion and transplantation therapy.

1. Antigen inheritance. ABO genes are inherited in a codominant manner following simple Mendelian genetics laws. Several other genetic loci interact with the ABO locus: H, Se, Le, I, and P. ABO group inheritance is controlled by the A, B, and H genes. These genes code not for the antigens themselves but for the production of glycotransferases, which are involved in the formation of their respective antigenic determinants. (Web Color Image 6–3.)
   a. H gene: L-fucose
   b. A gene: N-acetylgalactosamine
   c. B gene: D-galactose

2. Antigen development
   a. H antigens are precursors for the A and B antigens. A, B, and H antigens may be found in other body secretions, including saliva, urine, tears, amniotic fluid, milk, bile, exudates, and digestive fluids. The presence of H substances in body
secretions is controlled by the \textit{Se} gene. Individuals who are homozygous (Se Se) or heterozygous (Se se) for this gene are called secretors (approximately 80% of the population).

1. Group O secretors have H antigen in their secretions.

2. Group A secretors have A and H antigens in their secretions.

3. Group B secretors have B and H antigens in their secretions.

4. Group AB secretors have A, B, and H antigens in their secretions.

b. Development. A and B antigens begin to develop in the sixth week of fetal life, but do not reach adult levels until 3 years of age. Antigen levels approximate 50% at birth.

c. Because the O gene does not result in the conversion of H antigen to other antigens, the RBCs of group O individuals have the highest concentration of H antigen. Group AB individuals have the lowest concentration.

3. Phenotypes. ABO genes allow for five different phenotypes. Table 6–1 shows the frequency of the ABO phenotypes.

a. A phenotype. Group A individuals may be homozygous (AA) or heterozygous (AO). They have A antigens on their RBCs and anti-B antibodies in their serum or plasma. The two main subgroups, A1 and A2, can be differentiated by using the lectin anti-A1 reagent made from \textit{Dolichos biflorus} seeds.

(1) This reagent agglutinates RBCs with the A1 antigen, but not cells with the A2 antigen.

(2) Approximately 80% of group A individuals are A1; approximately 20% are A2.

(3) Anti-A1 can be found in 1% to 8% of group A2 individuals.

(4) Several other subgroups of A exist, but are extremely rare. The most common, A3, shows mixed field agglutination with anti-A or anti-AB reagents.

b. B phenotype. Group B individuals may be homozygous (BB) or heterozygous (BO). They have B antigens on their RBCs and have anti-A antibodies in their serum or plasma. A few weak subgroups of B exist, but are less common than the rare A subgroups.

c. AB phenotype. Group AB individuals have both A and B antigens on their RBCs, but lack both anti-A and anti-B antibodies in their serum or plasma.

d. O phenotype. Group O individuals are homozygous (OO) and have neither A nor B antigens on their RBCs. They have both anti-A and anti-B antibodies in their serum or plasma. Anti-AB antibodies are also found in the serum or plasma of Group O individuals.

e. Bombay phenotype. Although the H gene is necessary for the development of A and B antigens, some individuals lack the H gene and are homozygous for the \textit{h} gene, which results in the absence of A, B, or H antigens. These individuals type initially as group O. However, and reverse typing, they react strongly not only to A1 and B cells but also to O cells. Confirmatory testing is done using an anti-H reagent made from the \textit{Ulex europaeus} plant. The Bombay phenotype is very rare; fewer than 200 cases have been documented worldwide.

f. ABO antibodies are mostly naturally occurring antibodies that develop shortly after birth following exposure to ABO-like antigens in the environment. They are mostly IgM and react best at room temperature or below. Immune ABO antibodies develop in response to exposure to ABO-incompatible RBCs or other sources of exposure to ABO antigens. The immune ABO antibodies are usually IgG and can cross the placental barrier.
5. Testing
   a. **Forward grouping** analyzes patient cells for the presence of ABO antigens. Testing is performed at room temperature by adding anti-A and anti-B reagents to the patient’s RBCs. Cell suspensions used in blood banking procedures are usually 3% to 5%. All group O donors should also be tested with anti-AB reagent. Agglutination is a positive reaction.
   b. **Reverse grouping** analyzes patient serum or plasma for the presence of anti-A or anti-B antibodies. In adults, anti-A or anti-B is present in the serum when the corresponding antigen is not present on the RBCs. Because neonates have not developed these types of antibodies, they are not candidates for reverse ABO grouping. Testing is performed at room temperature by adding the patient’s serum or plasma to suspensions of known A₁ and B RBCs. Agglutination is a positive reaction.
   c. **Discrepancies** may occur in ABO testing for a variety of reasons, including:
      (1) **Subgroups of A or B.** Individuals with subgroups of A or B forward type as group O, but reverse type as group A or B. This discrepancy can be resolved by adsorbing and eluting anti-A or anti-B from the RBCs.
      (2) **Polyagglutination** can be caused by Tn activation or acquired B phenomenon. Tn activation occurs when a somatic mutation exposes a hidden RBC antigen. These discrepancies can be resolved by determining if the cells are Tn activated or have the acquired B antigen.
      (3) **Interfering substances in the serum or plasma.** False-positive agglutination can result from such substances as Wharton’s jelly or increased serum proteins. The patient will forward type as AB. This discrepancy can be resolved by thoroughly washing the patient’s RBCs and repeating the test.
      (4) **IgM alloantibodies.** Some IgM antibodies such as anti-Lea, anti-P, anti-M, and anti-N can cause false-positive results. Reverse-grouping reagent cells may have the antigens against which the alloantibodies are formed. Patients may forward type as A or B, but reverse type as O. This discrepancy can be resolved by identifying the alloantibody and retesting with reagent cells that lack the antigen.
      (5) **Strong autoantibodies.** Autoantibodies, such as anti-I, may cause a patient to forward type as A or B, but reverse type as O. This discrepancy can be resolved by autoabsorbing the serum and using it to repeat the testing.
      (6) **Rouleaux** caused by increased serum proteins (e.g., myeloma proteins) can cause a patient to forward type as AB and reverse type as O. This discrepancy can be resolved by performing a saline replacement to repeat the testing.
      (7) **The age of the patient** can cause discrepancies in ABO typing. Neonates do not have ABO antibodies and cannot be reverse typed, which makes it impossible to confirm the forward typing. Elderly patients may have weakened levels of ABO antibodies.
      (8) **Disease.** Certain diseases associated with hypogammaglobulinemia or agammaglobulinemia can result in patients reverse typing as AB.

B. **The Rh blood group system** is one of the most complex systems, because nearly 50 different Rh antigens have been identified.

1. **Nomenclature.** Four different nomenclature systems have been developed to identify the Rh antigens and to describe phenotypes. Table 6–2 and Table 6–3 compare the nomenclature and frequency of the most common Rh phenotypes.
   a. **Fisher-Race.** Rh antigens are inherited as three closely linked sets of alleles with little or no crossing over between loci. The five major Rh antigens are defined as D, C, E, c, and e.
   b. **Wiener.** Rh antigens are inherited as products of single genes at single loci. The five major Rh antigens are defined as Rhₐ, rh', Rhₐ', hr, and hr', which correspond to D, C, E, c, and e, respectively.
   c. **Rosenfield.** Rh antigens are assigned numbers to correspond to antigens already designated by other nomenclature. Newer antigens are numbered based on the
Table 6–2 Most Common Rh Phenotypes and Their Frequencies

<table>
<thead>
<tr>
<th>Phenotype Nomenclature</th>
<th>Frequency (%)</th>
<th>Fisher-Race</th>
<th>Weiner</th>
<th>Rosenfield</th>
<th>Whites</th>
<th>Blacks</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDe Rh1 Rh: 1,2, -3, -4,5</td>
<td>42</td>
<td>17</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cde rh Rh: -1, -2, -3,4,5</td>
<td>37</td>
<td>26</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cDE Rh2 Rh: 1, -2,3,4,-5</td>
<td>14</td>
<td>11</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cDe Rh0 Rh: 1, -2, -3,4,5</td>
<td>4</td>
<td>44</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

order of their discovery. The five major Rh antigens are defined as Rh1, Rh2, Rh3, Rh4, and Rh5, which correspond to D, C, E, c, and e, respectively.

d. International Society of Blood Transfusion (ISBT). Numeric terminology where each known system is given a number; and each antigen within the system is numbered sequentially in order of discovery.

(1) Six digit number for each authenticated blood group specificity (i.e., 004001)
(2) First three numbers represent system (004 = Rh blood group system)
(3) Last three numbers antigenic specificity (001 = D antigen within Rh blood group system)

2. D antigen is the most clinically significant of all non-ABO antigens. It is so highly immunogenic that a single exposure to D-positive blood results in the formation of anti-D antibodies in more than 50% of D-negative individuals.

a. The D antigen is the only Rh antigen that undergoes routine testing, except in the case of investigation of unexpected antibodies.

b. The D antigen is comprised of several component antigens that are inherited as a group. Incomplete D antigens result when some of the component parts are missing. Individuals with these incomplete D antigens are called D mosaics.

c. D^* is a weakened form of the D antigen. D-negative donors and obstetric patients must be tested for D^*. D^*-positive individuals are classified as D positive in blood donor testing. D^*-positive recipients should always receive D-negative blood. A D-negative, D^*-positive infant born to a mother with anti-D in her serum can suffer from severe hemolytic disease of the newborn (HDN) (see Section X). D^* can be inherited three ways:

(1) As an incomplete antigen (D mosaic)
(2) Due to position effect, which results in steric hindrance
(3) As a result of genetic coding for weakened D expression

3. The genes for C and c antigens are codominant. These antigens are less immunogenic than D antigens. Other alleles can be inherited in place of C or c at the C locus, and although they typically occur with low frequency, the antibodies stimulated by these antigens may sometimes be clinically significant. However, the e-like antigen (Rh26) is seen often and is found on almost all c-positive RBCs.

4. E and e antigen expression is codominant. E antigens are almost as immunogenic as D antigens, although e antigens are the least immunogenic of the five major Rh antigens.

5. G antigens are produced by the same Rh gene complexes that produce C and D antigens. Most C-positive and D-positive RBCs are also G-positive.

6. Compound antigens occur when two Rh genes are on the same chromosome. The five compound antigens of the Rh system are:

Table 6–3 Most Common Antigens of the Rh Blood Group in Four Nomenclatures

<table>
<thead>
<tr>
<th>Numeric</th>
<th>Fisher-race</th>
<th>Weiner</th>
<th>ISBT Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rh1</td>
<td>D</td>
<td>Rhb</td>
<td>004001</td>
</tr>
<tr>
<td>Rh2</td>
<td>C</td>
<td>rh</td>
<td>004002</td>
</tr>
<tr>
<td>Rh3</td>
<td>E</td>
<td>rh'</td>
<td>004003</td>
</tr>
<tr>
<td>Rh4</td>
<td>c</td>
<td>hr</td>
<td>004004</td>
</tr>
<tr>
<td>Rh5</td>
<td>e</td>
<td>hr'</td>
<td>004005</td>
</tr>
<tr>
<td>Rh6</td>
<td>ce</td>
<td>hr</td>
<td>004006</td>
</tr>
</tbody>
</table>
a. f (ce)

b. rh1 (Ce)

c. cE

d. CE

e. V (ces)

7. D-deletion genes lack alleles at the Ee locus or the Ee and Cc loci and are associated with unusually strong D antigens.

8. Rhnull RBCs lack all Rh antigens including Rh29. Rh29 is present on all RBCs except Rhnull individuals and is the highest incidence antigen in the Rh system. The lack of RBC antigens results in the characteristic stomatocytes seen in Rhnull individuals, who also suffer from a compensated hemolytic anemia.

9. LW antigens are rarely clinically significant, but must be considered when a D-positive person appears to have developed an anti-D antibody. Anti-LW reacts more strongly with D-positive than D-negative cells. Of the three alleles, the most common is LW*. LW* is encountered much less frequently, and the third allele, LW, is silent.

10. Rh-system antibodies are usually RBC-stimulated, either through transfusion or during pregnancy. Most are IgG1 or IgG3, and appear between 6 weeks and 6 months after exposure to the Rh antigen.

a. They do not agglutinate in saline unless there is a major IgM component.

b. They react best at 37°C and can be demonstrated by testing in high-protein media or by the indirect antiglobulin test.

c. Reaction is enhanced by the use of enzyme-treated RBCs.

d. They do not usually bind complement.

e. Rh antibodies often occur together, for example, anti-D and anti-G, or anti-Ce and anti-C.

f. They cross the placenta and can cause HDN.

11. Rh-antigen-detection reagents. There are several types of reagents used to detect Rh antigens.

a. High-protein IgG anti-D reagents are the most commonly used and require the use of an anti-D control.

b. IgM anti-D reagents are used in immediate-spin saline testing and are not suitable for D* testing.

c. Chemically modified IgG anti-D reagents may be used in direct saline agglutination testing.

d. Monoclonal anti-D reagents are combinations of monoclonal IgM and IgG, which can detect Rh antigens both at immediate spin and in the antiglobulin test phase.

e. Reagents are also available to test for C, c, E, and e antigens.

f. They do not usually bind complement.

g. Rh antibodies often occur together, for example, anti-D and anti-G, or anti-Ce and anti-C.

h. They cross the placenta and can cause HDN.

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e. Reagents are also available to test for C, c, E, and e antigens.

f. They cross the placenta and can cause HDN.

12. Testing is optimal at 37°C. If the RBCs are coated with immunoglobulin, the immunoglobulins may first be heat eluted, and the cells are then tested with saline reagents.

a. False-positive reactions may be caused by:

   (1) Positive direct antiglobulin test (DAT). The most common cause of Rh-typing discrepancies is a positive DAT. This type of discrepancy can be resolved by using low-protein reagents.

   (2) Rouleaux [see Section II.A.5.c(6)]

   (3) Cold autoagglutinins. False-positive reactions may occur because of the presence of cold autoagglutinins. The patient sample should be warmed to 37°C and immediately retested. The autoantibody should be investigated and identified.

b. False-negative reactions may be caused by:

   (1) Incorrect cell suspension. False-negative reactions can occur if the patient RBC suspension is too concentrated.

   (2) Improper procedure. Failure to follow the manufacturer’s directions can result in false-negative results.

C. The Lewis blood group system

1. Antigens in the Lewis blood group system do not develop as integral parts of the RBC membrane, but are adsorbed by the RBCs from the surrounding plasma.
a. Expression. The amount of antigen present is variable and is partially dependent on the individual's ABO phenotype and age, because Lewis antigens develop gradually. Lewis antigen expression is affected by H, Se, and Le genes. Lewis antigen expression may decrease dramatically during pregnancy.

b. The most common phenotypes are:
   (1) Le (a+b−)
   (2) Le (a−b+)
   (3) Le (a+b+)
   (4) Le (a−b−)

c. Most neonates type as Le (a−b−) regardless of which Lewis genes they have inherited.

2. Lewis antibodies
   a. Anti-Lea antibodies are usually IgM, but may also be IgG in total or in part.
      (1) Most of these antibodies react best at room temperature, but may react at 37°C.
      (2) Although most Lea antibodies are considered clinically insignificant, they can bind complement and are, therefore, capable of triggering in vitro hemolysis. Although Lea antibodies are not associated with HDN, the antibodies that react at 37°C or that cause in vitro hemolysis may be associated with hemolytic transfusion reactions (HTR).
      (3) Anti-Lea activity is enhanced by enzyme treatment.
   b. Anti-Leb antibodies are usually IgM and react best at room temperature. They bind complement poorly. Anti-Leb activity is enhanced by enzyme treatment. Leb antibodies are not associated with HDN and rarely cause HTR.
   c. Lewis antibodies may appear transiently during pregnancy in Le (a−b−) women, but disappear after delivery.
   d. Lewis antigens neutralize Lewis antibodies. Because both anti-Lea and anti-Leb react with most cells on routine RBC panels, Lewis substance can be used to neutralize the antibodies and allow the detection of any other antibodies present.

D. The MNS blood group system

1. Antigens
   a. The MNS antigens are determined by the MN and Ss loci; MN is associated with glycophorin A; Ss is associated with glycophorin B.
   b. There are five principal antigens in the MNS system: M, N, S, s, and U. RBCs with the S or s antigen also have the U antigen.
   c. MNS antigens are important markers in paternity studies.

2. Antibodies
   a. Anti-M antibodies are relatively common. They are usually naturally occurring and may be both IgM and IgG. Anti-M antibodies do not bind complement and react optimally at room temperature or below. They are only rarely associated with HDN or HTR.
   b. Anti-N antibodies are rare. They are weak, naturally occurring IgM antibodies that react best at room temperature or below. They are not usually associated with HDN or HTR.
   c. Anti-S, anti-s, and anti-U antibodies are rare. These IgG antibodies usually develop following RBC stimulation, and all have been associated with severe HDN and HTR. Although S, s, and U antibodies are usually reactive in the antiglobulin phase of testing, some saline reactive antibodies have been reported.

3. Testing
   a. In addition to human, rabbit, and monoclonal serum-typing reagents, lectin reagents are also used to test for M and N antigens. Examples include Vicia graminia and several Bauhinia species for anti-N, and several Iberis species for anti-M.
   b. Many M, N, and S antibodies demonstrate a dosage effect; that is, they react more strongly with homozygous than heterozygous cells.
   c. M and N antigens are destroyed by enzyme treatment, whereas S, s, and U are not as easily destroyed or have no effect.
E. The P blood group system

1. Antigens. P blood group antigens are structurally related to ABO antigens and exist as glycoproteins and glycolipids. There are five phenotypes:
   a. P1 is one of the two most common phenotypes. Approximately 75% of the population possesses the P1 phenotype. P1 individuals have both P and P1 antigens. The P antigen is well developed at birth; the P1 antigen is poorly developed at birth.
   b. P2 is the other most common phenotype. Individuals with this phenotype have only P antigen on their RBCs.
   c. P1 phenotype is very rare. Individuals with this phenotype have both P1 and P antigens on their RBCs.
   d. P2 phenotype is also very rare. Individuals with this phenotype have both the P2 and P antigen on their RBCs.
   e. The p phenotype is extremely rare. The RBCs of individuals with this phenotype are negative for P, P1, and P2 antigens.

2. Antibodies
   a. Anti-P1 antibodies are naturally occurring, cold-reacting, IgM antibodies often seen in individuals with the P2 phenotype. They rarely react at higher than room temperature, but they do bind complement. Anti-P1 antibodies do not cause HDN and are rarely associated with HTR.
   b. Anti-P antibodies are produced by individuals with P1 or P2 phenotypes and can trigger severe HTR. Autoanti-P (the Donath-Landsteiner antibody) is a cold-reacting antibody associated with paroxysmal cold hemoglobinuria (PCH).
   c. Anti-PP, Pk, and anti-p antibodies occur only rarely.

F. The I blood group system

1. Antigens. The I blood group antigens are structurally related to the ABO antigens and are found on RBC membranes as well as in plasma, milk, and amniotic fluid. Both I and i antigens are found on all RBCs. I antigens are poorly developed at birth. As the I antigenic strength increases, i antigen strength decreases. Most adults have strong I antigen expression and weak i antigen expression.

2. Antibodies
   a. Anti-I antibodies are naturally occurring, cold-reacting IgM antibodies. They fail to react with cord RBCs. Some anti-I antibodies react in a broader temperature range and can cause cold-agglutinin disease (CAD). CAD may be idiopathic or may be associated with diseases, such as *Mycoplasma pneumoniae* infections. Anti-I antibodies are not associated with HDN.
   b. Anti-i antibodies are rare. Like anti-I, they are cold-reacting antibodies. They may be seen in cases of infectious mononucleosis and may cause an associated hemolytic anemia that disappears as the infection resolves.

3. Testing for anti-I or anti-I antibodies is done at 4°C using group O RBCs or cord RBCs.

G. The Duffy blood group system. The Duffy system (FY) has four alleles that are responsible for the major antigens and resulting phenotypes: Fya, Fyb, and Fy. Fy is a weakened form of Fyb, and the Fy allele produces no gene product. There are four phenotypes:
   a. FY (a+b−)
   b. FY (a−b+)
   c. FY (a+b+)
   d. FY (a−b−) (There is a high incidence of this phenotype among blacks. The RBCs of individuals with this phenotype are resistant to infection by *Plasmodium vivax*.)

2. Antibodies. The most common FY antibodies are Fya and Fyb, which are both commonly encountered in blood banks. Other Duffy antibodies are very rare. Anti-Fya and anti-Fyb antibodies are usually IgG, often bind complement, and generally react only at the antiglobulin phase of testing. Both are destroyed by proteolytic enzyme treatment and heating to 56°C. Anti-Fya antibodies are seen more frequently than Fyb antibodies, and both occur more commonly in combination with other RBC antibodies
than alone. Although both can cause delayed HTR, only Fya has occasionally been implicated in HDN.

**H. The Kell blood group system**

1. **Antigens.** The Kell (K) system is comprised of 21 high- and low-incidence antigens, the most significant of which are K and k. The K0 or Knull phenotype lack all Kell antigens. An antigen associated with the Kell system, Kx, is located on the X chromosome. RBCs that lack the Kx antigen also have greatly weakened expression of the other Kell system antigens. These RBCs are morphologically acanthocytes, have decreased survival, and are less permeable to water. This syndrome, known as MacLeod syndrome, is also characterized by splenomegaly, reticulocytosis, and occasional association with chronic granulomatous disease (CGD). Kell antigens are destroyed or inactivated by sulfhydryl reagents.

2. **Antibodies.** The two most important antibodies of the Kell system are anti-K and anti-k (Cellano).
   a. The K antigen is second only to the D antigen in immunogenicity, and the resulting antibody is relatively common in transfusion practice. Finding K-negative donor units is rarely a problem, however, because more than 90% of the population is K-negative.
   b. Anti-K antibodies are usually IgG antibodies that react best at 37\(^\circ\)C and may occasionally bind complement. They can cause both HDN and HTR.
   c. Anti-k antibodies are rare but can cause both HDN and HTR.
   d. Antibodies to K0 (Knull) antigens are called anti-Ku or anti-KEL5 antibodies and are considered clinically significant. K0 patients should be transfused with K0 cells. In testing, K0 cells can be made by treating normal RBCs with 2-aminoethylisothiouronium (AET) bromide or dithiothreitol (DTT) plus cysteine-activated papain (ZZAP).

**I. The Kidd blood group system**

1. **Antigens.** There are two major antigens in the Kidd (JK) system, Jka and Jkb, which allows for four phenotypes:
   1. Jk(a\(^+\)b\(^−\))
   2. Jk(a\(^+\)b\(^+\))
   3. Jk(a\(^−\)b\(^+\))
   4. Jk(a\(^−\)b\(^−\))
   a. A third antigen, Jk3, is present on both Jka- and Jkb-positive RBCs. Both Jka and Jkb may exhibit dosage effect; reactivity may be enhanced by enzyme treatment.

2. **Antibodies.** Although Jka and Jkb antigens are poorly immunogenic, the resulting antibodies can cause severe HTR, and both are especially noted for causing delayed reactions. Both can occasionally cause mild HDN. Most Jka and Jkb antibodies are IgG1 or IgG3 and bind complement very efficiently. Both react best at 37\(^\circ\)C in the antiglobulin phase of testing, but saline-reactive antibodies may be seen. Jka and Jkb antibodies can be difficult to detect, and enhancement techniques, such as enzymes, low ionic strength saline (LISS), or polyethylene glycol (PEG), can help identify them.
antibodies react best at 37°C in the antiglobulin phase of testing. Lea antibodies can cause mild HTR, but cause mild HDN only rarely. Lea antibodies are often naturally occurring, whereas Leb antibodies usually develop after RBC stimulation by transfusion or pregnancy.

K. Other blood group systems
1. The Cartwright (Yt) system has two antigens, Yta and Ytb. The majority of individuals are Yt (a+). Antibodies are rare. If they develop, they are IgG antibodies that react best in the antiglobulin phase of testing and are not clinically significant.
2. The Colton (Co) system has three antigens, Coa, Cob, and Co-ab. Antibodies to Coa and Cob are rare, but can cause HTR. Neither cause HDN.
3. The Diego (Di) system has two antigens, Di-a and Di-b. Antibodies to both antigens are rare, although they have been reported as causes of rare cases of HDN. The Di-a antigen is useful as a racial marker; because it is seen almost exclusively in individuals of Mongolian extraction, such as North, Central, and South American Indians, Japanese, and Chinese.
4. The Dombrock (Do) system has two antigens, Do-a and Do-b. Antibodies are rare. Anti-Do-a antibodies can cause HTR, but not cause HDN.
5. The Scianna (SC) system has three antigens, SC1, SC2, and SC3. Antibodies to SC1 and SC2 are rare IgG antibodies that can be clinically significant.
6. The Xg system has only one antigen, Xga, and two resulting phenotypes: Xg (a+) and Xg (a−). The Xg-a antigen is X linked and is more common in women. The antigen is destroyed by enzyme treatment. Xg-a antibodies are usually IgG antibodies that bind complement but do not cause HDN or HTR.
7. The Cromer system is composed of eight high-incidence antigens (Cra, Tca, Tcab, Dr-a, Esa, Wesb, UMC, and IFC) and three low-incidence antigens (Tcb, Tcc, and Wesa). These antigens are located on the decay-accelerating factor on the RBC membrane. Patients with paroxysmal nocturnal hemoglobinuria have weakly expressed or absent Cromer-related antigens.

L. Miscellaneous blood group antigens. Several other RBC antigens have been identified, but have not been classified as part of any blood group system.
1. Bg antigens. There are three Bg antigens: Bga, Bgb, and Bgc. Bg antigens are related to human leukocyte antigens (HLA) on RBCs. Antibodies to Bg antigens are not clinically significant.
2. Sd-e antigens are high-incidence antigens found in several tissues and body fluids. Antibodies to these antigens are not clinically significant. Mixed-field agglutination is characteristically seen with Sd-e antibodies.
3. High-titer low-avidity (HTLA) antigens occur with high frequency, but the resulting antibodies are very weak and have little or no clinical significance. They do not cause HDN or HTR. There are several HTLA antigens:
   a. Chido (Ch-a) and Rodgers (Rga) genes are linked to HLA genes, and the antigens are associated with the C4d component of complement. Both antigens are denatured by proteolytic enzymes.
   b. Cost-Sterling (CSa) and York (Yka) antigens
   c. Knops (Kna) and McCoy (McCa) antigens
4. Independent high-frequency antigens rarely produce antibodies.
   a. Gregory (Gya) and Holley (Hya) antigens are IgG antibodies that can cause accelerated red cell destruction. They do not cause HDN.
   b. The Gerbich collection of antigens includes five antigens. Antibodies are extremely rare but may cause HDN or accelerated RBC destruction.
   c. Vel antigens. Antibodies to Vel antigens may cause HTR, but not HDN. They are usually IgG and can bind complement.
5. Independent low-frequency antigens very rarely cause antibody formation. Such antibodies are not clinically significant. Table 6-4 outlines the frequency of the most common blood group antigens.
Table 6–4 Frequency of Common Blood Group Antigens

<table>
<thead>
<tr>
<th>Antigen</th>
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<tr>
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<tr>
<td>Leb</td>
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<td>55</td>
</tr>
<tr>
<td>M</td>
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</tr>
<tr>
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</tr>
<tr>
<td>Jk*</td>
<td>72</td>
<td>43</td>
</tr>
</tbody>
</table>

III. DONOR SELECTION AND BLOOD COLLECTION

A. Blood collection for homologous donors begins with donor selection. The careful screening of donors ensures that the donation will not harm the donor and the donated blood will not harm the recipient. Certain guidelines established by the Food and Drug Administration (FDA) and the American Association of Blood Banks (AABB) are followed to help establish the safest possible blood supply. Following is a brief description of these guidelines:

1. Age. In general, donors should be between 17 and 65 years of age.

2. General appearance. The donor should appear to be in good health.

3. Weight. Donors should weigh at least 110 pounds to donate a full 450-mL unit. Donors weighing less than 110 pounds should have proportionately less blood drawn.

4. Temperature measured orally should not exceed 37.5°C (99.5°F).

5. Pulse should be between 50 and 100 beats per minute.

6. Blood pressure. Systolic pressure should be no higher than 180 mm Hg; diastolic pressure should be no higher than 100 mm Hg.

7. Hemoglobin and hematocrit. Blood for hemoglobin and hematocrit determination may be collected by venipuncture or fingerstick.

a. By venipuncture or fingerstick, the hemoglobin and hematocrit measurements should be no lower than 12.5 g/dL and 38%, respectively.

8. Time between donations. At least 8 weeks should lapse between whole blood donations; 16 weeks after 2-unit red cell collection; 4 weeks after infrequent apheresis, >2 days after plasma, platelet (not to exceed 24 times/year) or leukapheresis.

9. Temporary deferrals. Certain conditions are cause for temporary deferrals and include:

a. Having had a blood transfusion in the past 12 months
b. Pregnancy. AABB mandates existing pregnancy or pregnancy in the past 6 weeks as cause for deferral for 6 months.
c. Undergoing tattooing or ear piercing in the past 12 months
d. Having had close contact in the last 12 months with a person with viral hepatitis
e. Malaria. 12-month deferral for travel to endemic area, 3-year deferral for diagnosis of malaria or lived 5 consecutive years in endemic area.
f. Leishmania Risk. Travel to Iraq in the last 3 years, 12-month deferral from last departure
g. West Nile Virus (WNV). 120-day deferral from symptoms of headache with fever during defined risk season or 120 days from recovery with a clinical diagnosis or suspicion of WNV infections (based on symptoms and/or lab testing)
h. Having received one of the following vaccinations:
10. Permanent deferrals are caused by the following conditions listed by the AABB:

a. Anyone who has ever used intravenous drugs (illegal IV drugs)

b. Men who have had sexual contact with other men since 1977

c. Anyone who has ever received clotting factor concentrates

d. Anyone with a positive test for HIV (AIDS virus)

e. Men and women who have engaged in sex for money or drugs since 1977

f. Anyone who has had hepatitis since his or her 11th birthday

g. Anyone who has had babesiosis or Chagas disease

h. Anyone who has taken Tegison for psoriasis

i. Anyone who has risk factors for Creutzfeldt-Jakob disease (CJD) or who has an immediate family member with CJD

j. Anyone who has risk factors for variant CJD

k. Anyone who spent 3 months or more in the United Kingdom from 1980 through 1996

l. Anyone who has spent 5 years in Europe from 1980 to the present.

11. Other questions concerning a donor’s medical history may reveal underlying medical problems that could be cause for deferral. Any case in question should be referred to the donor center’s medical director. Most prescription and over-the-counter medications are not cause for deferral, although any questions should be referred to the medical director.

B. Apheresis donors. The same selection criteria that apply to whole blood homologous donors apply to apheresis donors. Additionally:

1. Plasmapheresis donors must have a serum total protein of at least 6.0 g/dL. A serum protein electrophoresis should be performed in these individuals every 4 months. There should also be at least 48 hours between donations.
2. Plateletpheresis donors should have a platelet count of at least $150 \times 10^9/L$. Aspirin ingestion within 3 days of donation is cause for temporary deferral. There should be at least 8 weeks between donations.

3. Leukapheresis donors must have an absolute granulocyte count of at least $4 \times 10^9/L$.

4. Therapeutic apheresis may be performed for certain conditions, such as Goodpasture’s syndrome, thrombotic thrombocytopenic purpura, myeloid leukemia with hyperleukocytosis, and acute complications of sickle-cell disease.

C. Criteria for autologous donors are not as well defined or regulated as are those for homologous donors. Some general guidelines for autologous donor suitability have been established by the AABB, but the facility medical director should make final decisions. In general, donors should have a hemoglobin of at least 11.0 g/dL and should not have signs or symptoms of active infection. Blood should not be donated within 72 hours of scheduled surgery or transfusion.

D. Donor reactions. Although rare, some blood donors experience donation-related reactions, which range from mild to severe.

1. Symptoms include:
   a. Pallor
   b. Sweating
   c. Dizziness
   d. Nausea and vomiting
   e. Twitching and muscle spasms
   f. Loss of consciousness

2. True convulsions are extremely rare.

3. Phlebotomy should be discontinued immediately and the donor should be treated symptomatically.

E. Anticoagulants and preservatives. Several anticoagulants are currently approved by the FDA for storage of blood and blood components. FDA approval for anticoagulants and additive solutions requires that 70% of transfused RBCs must be present and viable 24 hours after transfusion.

1. The purposes of anticoagulants and preservative solutions are:
   a. Preventing physical changes to maintain the viability and function of the blood constituents
   b. Preventing bacterial contamination
   c. Minimizing cell lysis

2. Citrate-phosphate-dextrose (CPD) can be used to store blood for 21 days when stored between 1°C and 6°C. The citrate functions as an anticoagulant by binding calcium. The phosphate helps to increase adenosine triphosphate (ATP) production, and the dextrose provides energy for the RBCs.

3. The adenine in citrate-phosphate-dextrose-adenine (CPDA-1) provides a substrate for ATP production. The shelf life of blood collected in CPDA-1 is 35 days.

4. Although not used for routine blood collection, heparin may be used as an anticoagulant. Because heparin has no preservative qualities, blood collected in heparin must be transfused within 24 to 48 hours, preferably within 24 hours.

5. Stored RBCs may be rejuvenated up to 2 to 3 days after their expiration date by the addition of a solution containing pyruvate, inosine, glucose, and phosphate. They may be either washed and transfused within 24 hours or glycerolized and frozen.

IV. DONOR PROCESSING

A. Homologous donor processing. The FDA and AABB require that certain tests be performed on all blood units collected for homologous transfusion. All reagents used for donor blood processing must be FDA approved, and accurate and thorough record keeping is critical.
1. **ABO and RH typing.** Forward and reverse ABO typing must be performed on each donor unit. The Rh type must be determined using anti-D and an appropriate reagent control. All units that are initially anti-D negative must have a D* test performed. If the D* test is positive, the unit is labeled Rh positive. If the D* test and the Rh control are negative, the unit is labeled Rh negative.

2. **Antibody screen.** Although the AABB requires that screens for unexpected antibodies be performed only on donors with a history of transfusion or pregnancy, most blood centers perform antibody screens on all donors. The method used must be able to demonstrate clinically significant antibodies.

3. **Serologic tests for syphilis.** The FDA requires that a serologic test for syphilis be performed on all donor units.

4. **Hepatitis.** All donor units must be tested for hepatitis B surface antigen (HBsAg), hepatitis B core antigen (HBcAg), and hepatitis C (anti-HCV) and NAT for HCV RNA. Units that are repeatedly reactive for any of these markers must be discarded, and the donors must be permanently deferred.

5. **Alanine aminotransferase (ALT).** ALT testing is no longer required, but some donor centers still perform the test. It was required by European standards until the first part of 2005.

6. **Human immunodeficiency virus (HIV).** All donor units must be tested for HIV-1/2, antibody using an FDA-approved EIA method. If the initial screening test is negative, the unit is suitable for transfusion; if it is positive, the test must be repeated in duplicate. If any one of the duplicate tests is reactive, the unit must be discarded as well as any in-date components from prior donations. Confirmation tests for HIV include the Western blot and the immunofluorescence assay (IFA).

7. **Cytomegalovirus (CMV).** Testing for CMV is not required, but is often performed on donor units. The transfusion of CMV-negative units may be clinically indicated for low-birth-weight neonates, as well as organ or bone marrow transplantation patients, and other immunocompromised or immunodeficient patients. Box 6–1 summarizes the testing required for homologous donors.

8. **WNV.** Outbreaks of the WNV in the United States prompted donor testing for antibodies to WNV and, in 2003, NAT testing to detect RNA to the virus.

**B. Autologous donor processing.** Requirements for processing autologous donor units differ somewhat from those for homologous units.

1. **Testing.** In addition to ABO and Rh typing, testing for HBsAg, syphilis, and HIV-1 are required for processing autologous donor units. Most donor facilities perform all tests normally required for homologous units to allow unused autologous units to be used for other transfusion purposes.

2. **Labeling.** Donor units dedicated for autologous use must be clearly labeled “for autologous use only,” and a biohazard label must be attached to any unit repeatedly reactive for any of the previously mentioned tests for infectious diseases.

**Box 6–1 Testing Required for Homologous Donor Processing**

- ABO and Rh typing
- Antibody screen
- Serologic tests for syphilis
- Hepatitis B surface antigen (HBsAg)
- Hepatitis B core antibody (anti-HBc)
- Hepatitis C virus antibody (anti-HCV)
- HCV RNA
- HIV-1 RNA
- HIV-1 and HIV-2 antibody (anti-HIV-1 and anti-HIV-2)
- HIV p24 antigen
- HTLV-I and HTLV-II antibody (anti-HTLV-I and anti-HTLV-II)
- West Nile Virus (WNV RNA)
- Nucleic Acid Amplification Testing (NAT)
3. Storage. In addition to being stored between 1°C and 6°C, autologous units must be stored separately from homologous units.

V. BLOOD COMPONENTS AND COMPONENT THERAPY. A wide variety of blood components can be prepared from routinely drawn units of whole blood for many different therapeutic applications. Table 6–5 summarizes blood components and the clinical indications for their use.

A. Whole blood
1. Preparation. Normally, approximately 450 mL of blood are collected into a bag containing 63 mL of anticoagulant, usually CPD or CPDA-1.
2. The shelf life of whole blood collected in CPD is 21 days when stored between 1°C and 6°C. The shelf life is extended to 35 days when the blood is collected in CPDA-1 anticoagulant. A unit of blood must be transfused within 24 hours if the seal on the bag is broken to remove plasma. If the blood is not transfused in that time period, it must be disposed.
3. Therapeutic uses. Each unit of whole blood should increase the hematocrit from 3% to 5%, or the hemoglobin from 1 to 1.5 g/dL. There are few clinical indications for whole blood transfusions, although they may be appropriate for patients with rapid blood loss when increased volume, as well as increased RBC mass, is needed. Whole blood may also be used in exchange transfusions, especially in neonates.

B. Packed RBCs
1. Preparation. Each unit of packed RBCs contains approximately 250 mL. Packed cells are prepared by removing approximately 200 to 250 mL of plasma from a unit of whole blood. The cells may be prepared in an open system by allowing the cells to sediment, then removing the plasma. A closed system may also be used in which multiple bags are attached to the unit, and the plasma is expressed into one of the satellite bags. The hematocrit of RBCs separated by these methods should not exceed 70% to 80%.
2. Shelf life. Cells separated in an open system must be transfused within 24 hours. If the cells are separated in a closed system, they have the same expiration date as the original unit of whole blood. RBCs separated in a closed system with an additive bag

<table>
<thead>
<tr>
<th>Table 6–5 Blood Component Therapy</th>
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<tbody>
<tr>
<td>Component</td>
</tr>
<tr>
<td>Whole blood</td>
</tr>
<tr>
<td>Packed RBCs</td>
</tr>
<tr>
<td>Deglycerolized RBCs</td>
</tr>
<tr>
<td>Washed RBCs</td>
</tr>
<tr>
<td>Leukocyte-poor RBCs</td>
</tr>
<tr>
<td>FFP</td>
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</table>

DIC = disseminated intravascular coagulation; FFP = fresh frozen plasma; ITP = immune thrombocytopenia purpura; RBCs = red blood cells; WBC = white blood cell.
can have a second preservative solution added that will extend the shelf life to 42 days. They should be stored between 1°C and 6°C.

3. **Therapeutic uses.** The increase in hemoglobin and hematocrit in response to a unit of packed RBCs is the same as for whole blood. Packed RBCs are used to increase the RBC mass in patients who have symptomatic anemia.

C. **Deglycerolized frozen RBCs**

1. **Preparation.** RBCs to be frozen are collected in CPD, CPDA-1, or other additive systems and normally should be frozen within 6 hours. The cells are warmed and mixed with high molar concentrations of glycerol, then frozen at –65°C. Frozen units must be stored for up to 10 years. The cells must be deglycerolized before they can be transfused. 

Deglycerolization begins with thawing the cells at 37°C, then washing multiple times in a gradient concentration of saline, beginning with hypertonic concentrations and ending with an isotonic saline solution containing glucose. One unit of deglycerolized RBCs contains approximately 180 mL of cells.

2. **Shelf life.** Deglycerolized RBCs are stored between 1°C and 6°C and must be transfused within 2 hours of deglycerolization.

3. **Therapeutic uses.** The increase in hemoglobin and hematocrit in response to a unit is the same as for whole blood or packed cells. Freezing cells allows for long-term storage of rare donor units or autologous units. Transfusing deglycerolized RBCs also minimizes febrile or allergic reactions.

D. **Washed RBCs**

1. **Preparation.** Plasma is removed from whole blood after centrifugation, and the remaining RBCs are washed three times with 0.9% saline.

2. **Shelf life.** Washed RBCs have a shelf life of 24 hours after the original unit is opened, and they should be stored between 1°C and 6°C.

3. **Therapeutic uses.** One unit of washed RBCs increases the hemoglobin and hematocrit by the same amount as do unwashed cells. Washing RBCs removes most of the leukocytes and plasma from a unit of blood, which greatly reduces the risk of febrile or allergic (anaphylactic) reactions in susceptible patients. Washed RBCs are used for the rare patient who has anti-IgA antibodies because of IgA deficiency.

E. **Leukocyte-poor RBCs**

1. **Preparation.** Leukocyte-poor RBC preparations have at least 70% of the original white blood cells (WBCs) removed, and at least 70% of the original RBCs are left. There are several different methods of obtaining leukocyte-poor RBCs, including centrifugation, filtration, and washing.

2. **Shelf life.** If a closed preparation system is used, the shelf life is the same as the original unit of blood. The shelf life is reduced to 24 hours if an open system is used. Leukocyte-poor RBCs should be stored between 1°C and 6°C.

3. **Therapeutic uses.** In addition to increasing RBC mass, leukocyte-poor RBCs also minimize febrile transfusion reactions in patients who have leukocyte antibodies as well as reducing CMV transmission.

F. **Fresh frozen plasma (FFP)**

1. **Preparation.** Plasma is separated from whole blood and frozen within 6 hours of collection. Plasma can be removed from whole blood using a double bag collection system to preserve a closed system. The plasma is immediately frozen at or below –18°C.

2. **Shelf life.** After freezing, the plasma should be stored at or below –18°C. FFP has a shelf life of 1 year after collection of the original unit of blood. It should be thawed at 37°C and transfused within 24 hours of thawing. Thawed FFP should be stored between 1°C and 6°C if it is not transfused immediately.

3. **Therapeutic uses.** Because FFP contains all of the coagulation factors, it can be used to treat patients who have liver failure, vitamin K deficiency, and disseminated intravascular coagulation (DIC), or to patients who have received massive transfusions.
G. Platelets

1. Preparation. Platelet-rich plasma is separated at room temperature by centrifugation from RBCs within 6 hours of collection of whole blood. The platelet-rich plasma is then centrifuged, and the resulting platelet-poor plasma supernatant is removed, which leaves approximately 50 mL of plasma with the platelet concentrate.

2. Shelf life. Platelets are stored at room temperature with continuous gentle agitation and have a shelf life of 3 to 5 days, depending on the type of bag used. If several units of platelets are pooled, the shelf life is reduced to 4 hours following pooling.

3. Therapeutic uses. Platelet concentrate is used to treat patients who have thrombocytopenia, dysfunctional platelets, DIC, and idiopathic thrombocytopenia purpura (ITP), or to patients who have received massive transfusions. Each unit of platelet concentrate should increase the platelet count by 5,000 to 10,000/μL in a typical 70-kg human.

H. Plasma derivatives

1. Preparation. Plasma other than that prepared as FFP may be separated from whole blood at any time during the unit’s shelf life up to 5 days after the expiration date. The plasma may be pooled, purified, or fractionated into albumin or plasma protein fraction.

2. Shelf life. Plasma derivatives have a shelf life of 5 years when stored between 1 °C and 6 °C.

3. Therapeutic uses. Plasma derivatives such as albumin are used primarily as volume expanders.

I. Cryoprecipitate

1. Preparation. Cryoprecipitate is the insoluble fraction of plasma. Each unit contains 80 to 120 units of factor VIII and approximately 150 to 250 mg of fibrinogen, as well as significant amounts of factor XIII and fibronectin. Each unit of cryoprecipitate contains approximately 15 mL. It is prepared from FFP that has been partially thawed between 1 °C and 6 °C, centrifuged, and has had the supernatant removed. The remaining cryoprecipitate is immediately frozen at or below −18 °C.

2. Shelf life. After freezing, the optimal storage temperature is at or below −30 °C, and the shelf life is up to 12 months following the collection of the original unit.


J. Granulocytes

1. Preparation. Granulocyte preparations may be prepared by leukapheresis or from a freshly drawn donor unit.

2. The shelf life of granulocyte preparations is 24 hours after separation when stored at room temperature. However, granulocytes should be transfused as soon as possible because their half-life is only 6 hours.

3. Therapeutic uses. Granulocytes have been given to severely neutropenic patients or patients who have overwhelming sepsis. Success has been limited.

K. Irradiation of blood products. The immunologically active lymphocytes present in most blood components can create special problems for immunocompromised patients. Graft-versus-host disease (GVHD) is an especially serious complication for these patients. Irradiating blood products can help reduce the risk of GVHD and other related complications.

1. Indications for use. Irradiated blood products may be indicated for:
   a. Patients receiving chemotherapy or radiotherapy
   b. Organ transplantation recipients who have been immunosuppressed
   c. Low-birth-weight neonates
   d. Patients with genetically deficient immune systems

2. Irradiation
   a. Blood components should be irradiated immediately before transfusion.
   b. Doses of 1,500 to 5,000 rad are usually used.
   c. Expiration date of 28 days from the date of irradiation or the original outdate of the unit, whichever is sooner.
VI. ANTIGLOBULIN TESTING

A. Reagents. Patient RBCs are tested to detect the presence of in vivo and in vitro antibodies using antihuman globulin (AHG) reagents. These reagents may be monoclonal or polyclonal and may be monospecific or polyspecific.

1. Monospecific reagents are made against a specific immunoglobulin class or complement component. Anti-IgG is most commonly used because most clinically significant antibodies are IgG. These IgG reagents are directed specifically against the heavy chain component of the immunoglobulin molecule. Some monospecific reagents are specific for the C3b and C3d components of complement.

2. Polyspecific reagents are made against a combination of immunoglobulin classes and complement components, but always contain anti-IgG and anti-C3d.

B. Direct antiglobulin testing (DAT)

1. Application. DAT is used to demonstrate the in vivo coating of RBCs with antibody or complement. It may be used to investigate the following disorders:
   a. HDN (see Section X)
   b. Autoimmune and drug-induced hemolytic anemias
   c. HTR

2. Testing. Patient RBCs are saline washed and mixed with AHG reagents at room temperature, then centrifuged, resuspended, and examined for agglutination. Table 6–6 describes a typical system for grading agglutination reactions:
   a. Negative results are confirmed by the addition of Coombs' control cells, which should yield a positive reaction after centrifugation.
   b. Coombs' control cells are coated with IgG or C3d, depending on the specificity of the AHG reagent used. If the patient’s RBCs have been properly washed, the IgG or C3d on the control cells reacts with any antibody or complement bound to the surface of the patient’s RBCs.
   c. Patient RBCs should be collected in ethylenediamine tetraacetic acid (EDTA), acid-citrate-dextrose, or CPD anticoagulants.

3. False-positive reactions can be caused by:
   a. Dirty glassware
   b. Septicemia
   c. Overcentrifugation

4. False-negative reactions can be caused by:
   a. Inadequately washed RBCs (most common cause)
   b. Undercentrifugation
   c. Delayed testing after specimen collection

C. Indirect antiglobulin testing (IAT)

1. Application. The IAT is used to demonstrate the presence of antibody or complement in patient serum, which results in the in vitro coating of RBCs by the antibody or complement component. Uses of IAT include:

<table>
<thead>
<tr>
<th>Table 6–6</th>
<th>Agglutination Reactions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grade</td>
<td>Appearance</td>
</tr>
<tr>
<td>4+</td>
<td>One large aggregate of RBCs against a clear background</td>
</tr>
<tr>
<td>3+</td>
<td>Several large aggregates against a clear background</td>
</tr>
<tr>
<td>2+</td>
<td>Many small-to-medium aggregates against a clear background</td>
</tr>
<tr>
<td>1+</td>
<td>Many small aggregates against a cloudy background</td>
</tr>
<tr>
<td>±</td>
<td>Many very small, easily broken aggregates against a cloudy background</td>
</tr>
<tr>
<td>(+)</td>
<td>No agglutination visible macroscopically; agglutination visible microscopically</td>
</tr>
<tr>
<td>mf</td>
<td>Mixed field reaction; tiny aggregates against a background of free RBCs</td>
</tr>
<tr>
<td>0</td>
<td>Negative; no agglutination visible macroscopically or microscopically</td>
</tr>
</tbody>
</table>

RBCs = red blood cells.

*Negative or weakly positive reactions are read microscopically.
CHAPTER 6 Immunohematology

2. Testing. Patient serum is incubated at 37°C with RBCs (e.g., donor cells, antibody screening cells). After incubation, the cells are washed with saline to remove unbound antibody, and the AHG reagent is added. The AHG reagent reacts with the RBCs that have been sensitized by any antibodies present. Negative results are confirmed by the addition of Coombs' control cells, which should yield a positive result after centrifugation. Reactions may be enhanced by the use of one of the following reagents to the incubation phase of testing:

a. Bovine serum albumin at 22% or 30%, which increases the test’s sensitivity
b. LISS solutions, which also increase sensitivity and shorten incubation times
c. Other reagents to increase sensitivity, such as polybrene and PEG

3. False-positive reactions may be caused by:
   a. Polyagglutination resulting from in vivo coated RBCs (+ DAT)
   b. Overcentrifugation
   c. Dirty glassware

4. False-negative reactions may be caused by:
   a. Inadequately washed RBCs (most common cause)
   b. Undercentrifugation
   c. Delayed testing after specimen collection
d. Incorrect cell-serum ratio

VII. UNEXPECTED ANTIBODIES

A. Detection. Clinically significant unexpected antibodies against blood group antigens occur in less than 3% of the population. Antibodies occur more frequently in women than in men because of the possibility of sensitization during pregnancy. Multiple antibodies are more commonly seen in patients older than 60 years who have undergone transfusion multiple times. Table 6–7 summarizes the characteristics of clinically significant antibodies.

1. Screening cells are Group O cells that have known antigens present. Commercially available sets of screening cells contain D, C, E, c, M, N, S, s, Lea, Leb, P, K, k, Fya, Fyb, Jka, and Jkb antigens. Testing is performed in three consecutive phases using patient serum and screening cells:
   a. Immediate spin in saline at room temperature
   b. 37°C incubation with enhancement medium (e.g., albumin, LISS, polybrene)
c. AHG phase after incubated cells are washed with saline

2. An autocontrol (using patient cells and patient serum) is also performed. A negative result effectively rules out the presence of an autoantibody.

3. Results. IgM antibodies usually react on immediate spin and include Lea and Leb antibodies such as Kell, Rh, Kidd, and Duffy. IgG antibodies usually react in the AHG phase.

B. Antibody identification. Antibody panels are used to identify an unexpected antibody detected by antibody screening. These panels usually contain 10 to 15 vials of Group O cells, each of which yields a different antigen reaction pattern. Cord cells are useful in identifying cold antibodies because they strongly express the I antigen and weakly express the I antigen.

1. Testing. The patient’s serum is mixed with the panel cells and tested, along with an autocontrol, in the three consecutive phases:
   a. Immediate spin in saline at room temperature
   b. 37°C incubation with enhancement medium (e.g., albumin, LISS, polybrene)
c. AHG phase after the incubated cells are washed with saline
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Table 6–7 Characteristics of Clinically Significant Antibodies

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Class*</th>
<th>Phase†</th>
<th>HDN</th>
<th>HTR</th>
<th>Binds Complement</th>
</tr>
</thead>
<tbody>
<tr>
<td>D</td>
<td>IgG</td>
<td>AHG</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>C</td>
<td>IgG</td>
<td>AHG</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>E</td>
<td>IgG</td>
<td>AHG</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>e*</td>
<td>IgG</td>
<td>AHG</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Lea</td>
<td>IgM</td>
<td>RT</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Leb</td>
<td>IgM</td>
<td>RT</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>M</td>
<td>IgG, IgM</td>
<td>≤37°C</td>
<td>Rare</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>N</td>
<td>IgG, IgM</td>
<td>≤37°C</td>
<td>Rare</td>
<td>Rare</td>
<td>No</td>
</tr>
<tr>
<td>S</td>
<td>IgG, IgM</td>
<td>AHG</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>s</td>
<td>IgG</td>
<td>AHG</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>P1</td>
<td>IgM</td>
<td>4°C</td>
<td>No</td>
<td>Rare</td>
<td>Yes</td>
</tr>
<tr>
<td>Fya</td>
<td>IgG</td>
<td>AHG</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Fyb</td>
<td>IgG</td>
<td>AHG</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>K</td>
<td>IgG</td>
<td>AHG</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>k</td>
<td>IgG</td>
<td>AHG</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Jka</td>
<td>IgG</td>
<td>AHG</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Jkb</td>
<td>IgG</td>
<td>AHG</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Lua</td>
<td>IgM, IgG</td>
<td>RT</td>
<td>Rare</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Lub</td>
<td>IgM, IgG</td>
<td>AHG</td>
<td>Rare</td>
<td>Rare</td>
<td>Yes</td>
</tr>
</tbody>
</table>

AHG = antihuman globulin; HDN = hemolytic disease of the newborn; HTR = hemolytic transfusion reactions; RT = room temperature.

*Class = immunoglobulin class to which majority of antibodies belong.
†Phase = phase at which most of the antibodies react optimally.

2. Techniques to enhance antigen-antibody reactions may be used. These include:

a. Enzyme treatment with ficin, papain, trypsin, or bromelin to enhance the reactions of some antibodies (e.g., Rh and Kidd) or denature others (e.g., M, N, S, and Duffy) (Table 6–8)

b. Increasing the amount of serum to increase the number of available antibody molecules

Table 6–8 Effect of Proteolytic Enzymes on Antigen-Antibody Reactivity

<table>
<thead>
<tr>
<th>Antigen-Antibody</th>
<th>Reactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABO</td>
<td>Enhanced</td>
</tr>
<tr>
<td>Rh</td>
<td>Enhanced</td>
</tr>
<tr>
<td>Lea</td>
<td>Enhanced</td>
</tr>
<tr>
<td>Lea*</td>
<td>Enhanced</td>
</tr>
<tr>
<td>M</td>
<td>Destroyed</td>
</tr>
<tr>
<td>N</td>
<td>Destroyed</td>
</tr>
<tr>
<td>S</td>
<td>Destroyed</td>
</tr>
<tr>
<td>P1</td>
<td>Enhanced</td>
</tr>
<tr>
<td>i</td>
<td>Enhanced</td>
</tr>
<tr>
<td>Fya</td>
<td>No effect</td>
</tr>
<tr>
<td>Fyb</td>
<td>No effect</td>
</tr>
<tr>
<td>Jka</td>
<td>Enhanced</td>
</tr>
<tr>
<td>Jkb</td>
<td>Enhanced</td>
</tr>
<tr>
<td>K</td>
<td>No effect</td>
</tr>
<tr>
<td>k</td>
<td>No effect</td>
</tr>
<tr>
<td>Lua</td>
<td>Destroyed</td>
</tr>
<tr>
<td>Lub</td>
<td>Destroyed</td>
</tr>
<tr>
<td>Xga</td>
<td>Destroyed</td>
</tr>
</tbody>
</table>
Figure 6–1 Antibody panel in which a single alloantibody, Fy\(\alpha\), has been identified. Enzyme testing was not necessary.

3. **Other techniques** may be used to eliminate clinically insignificant reactions and make identification of significant antibodies easier. These include:
   a. **Enzyme treatment** as described previously
   b. **AET, DTT, and ZZAP**, which inactivate some antigens (especially Kell)
   c. **Thiol reagents**, which denature IgM immunoglobulins
   d. **Saline, LISS, albumin**
   e. **Increased or decreased incubation temperatures**
   f. **Saline replacement** to remove abnormal proteins
   g. **Adsorption and elution** to remove unwanted antibodies, such as cold or warm autoantibodies, or to help resolve multiple antibodies

4. **Titrations** may be used to detect the antibody present in the highest concentration in patients with multiple antibodies.

5. **Results.** The manufacturer’s sheet supplied with each antibody panel (Figure 6–1) indicates the presence or absence of specific antigens in each vial of panel cells. Results should be consistent with the characteristics of the identified antibody, such as optimal reaction temperatures.
   a. **Patient reactions** are recorded and compared to those in the panel, and antigens whose reactions do not match the patient’s reactions are eliminated until the antibody or antibodies are identified.
   b. **Hemolysis** should also be noted because some antibodies, especially those in the ABO, P, Le, Jk, and Vel systems, can cause hemolysis.

C. **Troubleshooting** (Table 6–9)
   1. If the autocontrol and all panel cells agglutinate at room temperature but react less strongly at 37°C and in the antiglobulin phase, then the presence of one of the following should be suspected:
      a. **Cold-reacting antibody** such as anti-I
      b. **Rouleaux**
   2. If the autocontrol and all panel cells agglutinate only in the antiglobulin phase, then the problem may be a **warm autoantibody**.
   3. If the autocontrol is negative, and all panel cells agglutinate, then there may be:
      a. **Multiple antibodies**
      b. **Antibody against a high-frequency antigen** found on all the panel cells
   4. If the autocontrol is positive in the antiglobulin phase, and the panel cells are negative or give variable positive reactions, then the problem may be a **warm AIHA**.
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Table 6–9 Troubleshooting Antibody Panels

<table>
<thead>
<tr>
<th>Room Temperature</th>
<th>37°C</th>
<th>AHG</th>
<th>Possible Problem</th>
</tr>
</thead>
<tbody>
<tr>
<td>Autocontrol</td>
<td>3+</td>
<td>1+</td>
<td>1+</td>
</tr>
<tr>
<td>Panel cells</td>
<td>3+</td>
<td>1+</td>
<td>1+</td>
</tr>
<tr>
<td>Autocontrol</td>
<td>0</td>
<td>0</td>
<td>+</td>
</tr>
<tr>
<td>Panel cells</td>
<td>0</td>
<td>0</td>
<td>pl</td>
</tr>
<tr>
<td>Autocontrol</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Panel cells</td>
<td>var</td>
<td>var</td>
<td>var</td>
</tr>
</tbody>
</table>

VIII. COMPATIBILITY TESTING

A. Patient specimens. Pretransfusion testing begins with a properly collected and identified patient specimen.

1. Patient identification. Proper identification of the patient is imperative; the patient must have a wristband with identification information. The tube should be labeled at bedside immediately after the sample is drawn. In addition to patient information, the label should also include the date and time of collection and the name of the person who collected the sample. A sample should never be collected into a previously labeled tube.

2. Collection. Serum is the preferred specimen for compatibility testing. Hemolysis should be avoided. Blood should not be drawn from an intravenous site unless absolutely necessary. In such a case, the infusion should be stopped, the line should be flushed with normal saline, and the first 5 to 10 mL of blood should be discarded before the specimen is collected.

3. Age of specimen. The freshest sample possible should be used for compatibility testing. If the patient has previously undergone transfusion or if the transfusion history is unknown, the sample should be no older than 72 hours. Pregnant patients should also be tested with samples not more than 72 hours old.

4. Sample storage. The AABB requires that patient samples must be stored between 1°C and 6°C for at least 7 days following transfusion.

B. Compatibility testing for homologous transfusion. The selection of donor units compatible for homologous transfusion includes:

1. ABO and Rh on donor units
2. ABO and Rh on recipient, including both forward and reverse ABO grouping (Rh is determined using anti-D reagents, and D\(^\text{+}\) testing may be performed on D-negative patients (although this is generally considered unnecessary, except in the case of obstetric patients)).
3. Antibody screening of recipient
4. Antibody identification. If an unexpected antibody is identified, it should be determined if the antibody is clinically significant (Table 6–10). If so, all donor units should be tested for that antibody, and only those units lacking the antigen should be used.
5. Autocontrol to detect the presence of autoantibodies
6. Crossmatch
   a. The major crossmatch tests donor cells against the recipient’s serum and primarily functions to determine the ABO compatibility of the donor cells.
   b. The minor crossmatch using recipient’s cells and donor serum is no longer required as part of the crossmatch procedure.
   c. The crossmatch is tested in three phases:
      1. Immediate spin in saline at room temperature
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Table 6–10 Clinically Significant Antibodies

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Frequently Significant</th>
<th>Sometimes Significant</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABO</td>
<td>L</td>
<td>L</td>
</tr>
<tr>
<td>Rh</td>
<td>l</td>
<td>l</td>
</tr>
<tr>
<td>Fya</td>
<td>L</td>
<td>L</td>
</tr>
<tr>
<td>Fyb</td>
<td>M</td>
<td>M</td>
</tr>
<tr>
<td>K</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>k</td>
<td>P₁</td>
<td>P₁</td>
</tr>
<tr>
<td>Jka</td>
<td>Jka</td>
<td>Jka</td>
</tr>
<tr>
<td>Jkb</td>
<td>Jkb</td>
<td>Jkb</td>
</tr>
<tr>
<td>S</td>
<td>s</td>
<td>s</td>
</tr>
</tbody>
</table>

(2) Incubation at 37°C with enhancement medium
(3) Antiglobulin phase after washing incubated cells with saline

C. Compatibility testing for autologous transfusion includes:
1. ABO and Rh on autologous units
2. ABO and Rh on recipient
3. Antibody screening and major crossmatch (not required, although an immediate-spin major crossmatch is often performed)

D. Compatibility testing for neonatal transfusion includes:
1. ABO and Rh on the infant
2. Antibody screen on the infant or the mother
   a. If the antibody screen is negative, a crossmatch is not necessary.
   b. If the donor cells are not group O, the infant must be tested for anti-A and anti-B antibodies. If either is present, ABO-compatible RBCs should be used. A crossmatch is not necessary.

E. Troubleshooting incompatible crossmatches (Table 6–11)

1. Negative antibody screen, negative autocontrol, and positive major crossmatch may be caused by:
   a. Incorrect ABO grouping of donor or recipient
   b. Donor unit with a positive DAT
   c. Donor with an alloantibody to a low-incidence antigen

2. Positive antibody screen, negative autocontrol, and positive major crossmatch may be caused by a recipient alloantibody to an antigen(s) on donor cells.

3. Positive antibody screen, positive autocontrol, and positive major crossmatch may be caused by:
   a. Recipient autoantibody
   b. Recipient alloantibody to recently transfused RBCs
   c. Rouleaux
      (1) Positive reactions are seen at 37°C.
      (2) Washing before the addition of AHG reagent should remove the excess protein that is causing the rouleaux formation.
      (3) The AHG phase should be negative.

Table 6–11 Troubleshooting Incompatible Crossmatches

<table>
<thead>
<tr>
<th>Antibody Screen</th>
<th>Autocontrol</th>
<th>Major Crossmatch</th>
<th>Possible Problem</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>Negative</td>
<td>Positive</td>
<td>ABO/Rh typing error, Donor unit with positive DAT, Low-incidence antibody in donor unit</td>
</tr>
<tr>
<td>Positive</td>
<td>Negative</td>
<td>Positive</td>
<td>Donor alloantibody</td>
</tr>
<tr>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
<td>Patient alloantibody, Rouleaux</td>
</tr>
</tbody>
</table>

DAT = direct antiglobulin test.
Table 6–12 Selection of Whole Blood Donor Units and Packed Red Blood Cells (RBCs) In Order of Preference

<table>
<thead>
<tr>
<th>Recipient ABO</th>
<th>Whole Blood</th>
<th>1st Choice</th>
<th>2nd Choice</th>
<th>3rd Choice</th>
<th>4th Choice</th>
<th>Packed RBCs</th>
</tr>
</thead>
<tbody>
<tr>
<td>O</td>
<td>O</td>
<td>O</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>A</td>
<td>A</td>
<td>A</td>
<td>O</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>B</td>
<td>B</td>
<td>B</td>
<td>O</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>AB</td>
<td>AB</td>
<td>AB</td>
<td>A</td>
<td>B</td>
<td>O</td>
<td>—</td>
</tr>
</tbody>
</table>

F. Selection of donor units according to ABO and Rh
1. The ABO group of the recipient (Table 6–12) is the most important consideration for selecting donor units for transfusion. Whenever possible, the donor units should be the same ABO group as the recipient. If this is not possible, the donor units must be ABO compatible with the recipient’s serum and must be given as packed RBCs.
2. The Rh type of the recipient is the second most important consideration.
   a. Rh-positive recipients may receive either Rh positive or Rh negative units.
   b. Rh-negative recipients should only receive Rh negative RBCs to avoid being sensitized to the D antigen. Rh-negative recipients may receive Rh positive RBCs if it has been demonstrated that anti-D is not present in the recipient.
3. Group O-negative RBCs are the component of choice for neonatal transfusions. Group-specific blood may be given if the mother and infant are the same ABO type. The donor RBCs must be compatible with the mother’s serum.
4. Emergency situations may call for the release of uncrossmatched blood. In such cases, O-negative or group-specific blood should be given, and compatibility testing should be performed as soon as possible.

IX. TRANSFUSION REACTIONS include any adverse signs or symptoms associated with a transfusion and may be acute or delayed.

A. Acute reactions may have immunologic or nonimmunologic causes.
1. Acute HTR are rare immunologic reactions that may be life-threatening and are usually caused by ABO incompatibilities. The associated hemolysis is intravascular. The most common signs of an acute hemolytic reaction are fever, chills, and hemoglobinuria. Dyspnea and hypotension leading to shock are seen in severe reactions. The most severe cases may result in DIC and renal failure.
2. Nonhemolytic febrile transfusion reactions are mild immunologic reactions that are caused by HLA class I antigens on transfused WBCs or platelets. They are the most common type of transfusion reaction (1 in every 200 transfusions). Fever and chills are the most common symptoms. The use of leukocyte filters when transfusing blood can help reduce the frequency of these reactions.
3. Allergic reactions are the second most common type of transfusion reactions. These acute immunologic reactions are typically associated with urticaria and are thought to occur in response to reactions between recipient antibodies and soluble proteins in the donor units. Antihistamines given before the transfusion can reduce the risk of an allergic reaction. Anaphylactic reactions are very severe allergic reactions. Although rare, they can be life-threatening. They are usually caused by antibodies to IgA.
4. Transfusion-related acute lung injury (TRALI) is a life-threatening transfusion reaction caused by HLA antibodies. Symptoms are the same as those seen in adult respiratory distress syndrome and include acute respiratory distress, hypoxemia, pulmonary edema, fever, and hypotension.
5. Acute nonimmunologic reactions may be caused by:
   a. Bacterial contamination of the blood product
   b. Circulatory overload caused by too rapid transfusion
c. Blood that has been hemolyzed by improper storage or mechanical stress (e.g., heart-lung machine)

B. Delayed reactions may also have immunologic or nonimmunologic causes.

1. Delayed HTR are characterized by the accelerated destruction of transfused RBCs and are most commonly associated with a secondary (amnestic) response to an RBC antigen. Delayed reactions may not be recognized for days, weeks, or even months after a transfusion, until a rapid decline in the recipient’s hematocrit is noticed. Patients may experience fever and mild jaundice. The associated hemolysis is generally extravascular. IgG antibodies to Rh, MNS, Kell, Kidd, and Duffy antigens are often implicated.

2. Transfusion-associated GVHD (TA-GVHD) reactions may be either acute or chronic. An extremely high mortality rate is associated with TAGVHD. These reactions occur when immunologically competent lymphocytes are transfused into an immunocompetent host. Bone marrow transplantation recipients may also develop TA-GVHD, but these patients typically have a chronic, milder disease with a much lower mortality rate. The transfusion of irradiated units can prevent TA-GVHD in immunocompromised patients.

3. Post-transfusion purpura is a rare transfusion reaction usually seen in older female patients who have been sensitized to platelet antigens, either by previous transfusion or pregnancy. These reactions are characterized by mild-to-severe immune thrombocytopenia with clinical bleeding in severe cases.

4. Other nonimmunologic transfusion reactions include:
   a. Citrate toxicity from anticoagulant
   b. Hypothermia from transfusing cold blood
   c. Hyperkalemia from the increased potassium levels in banked blood
   d. Transfusion-induced hemosiderosis (iron overload) in patients who have undergone chronic transfusion

C. Investigating transfusion reactions requires strict adherence to the following steps:

1. All patient records and blood product containers must be checked for proper identification. Clerical error is the most common cause of HTR.

2. A post-transfusion sample from the patient is centrifuged, and the serum or plasma is examined for icterus or hemolysis and compared against the pretransfusion sample.

3. A DAT is performed on a post-transfusion EDTA specimen. A DAT is performed on the pretransfusion specimen if the post-transfusion specimen has a positive DAT. An antigen-antibody incompatibility is assumed if the post-transfusion specimen DAT is positive, and the pretransfusion DAT is negative.

4. Other tests to further investigate the cause of the incompatibility may be performed, including:
   a. ABO and Rh on the recipient’s pretransfusion and post-transfusion specimens and on the donor units
   b. Repeat compatibility testing with posttransfusion and posttransfusion specimens
   c. Serum bilirubin on samples drawn 5 to 7 hours after transfusion
   d. Gram’s stain on recipient’s plasma and culture of donor unit bag
   e. Haptoglobin
   f. Urine hemoglobin
   g. Coagulation studies

X. HEMOLYTIC DISEASE OF THE NEWBORN (HDN) is sometimes referred to as erythroblastosis fetalis. It occurs when the mother is alloimmunized to antigen(s) found on the RBCs of the fetus, which results in the destruction of the fetal RBCs by the mother’s IgG antibodies. This hemolysis causes anemia in the fetus and anemia and hyperbilirubinemia in the newborn. ABO and Rh antibodies are most frequently implicated in HDN, although other alloantibodies (e.g., M, N, S, Kell, Duffy, Kidd, Lutheran) can also cause HDN. ABO-HDN is the most common form of the disease and is a milder disease than that caused by Rh antibodies. Anti-D, anti-CD, and anti-CE are associated with the
most severe forms of the disease. Anti-c, anti-E, and anti-k (Cellano) are associated with moderate forms.

A. Pathophysiology. If a woman’s first child has a RBC antigen(s) foreign to the mother, sensitization occurs during delivery, when the normal fetomaternal bleed allows some of the infant’s RBCs to enter the mother’s circulation. If subsequent fetuses have the same antigen(s) as the first, the mother’s IgG antibodies against that antigen cross the placental barrier and enter the fetal circulation. This secondary antibody response results in the hemolysis of the fetus’s RBCs, and the released hemoglobin is converted to bilirubin.

1. Before delivery, the glucuronyl transferase in the mother’s liver converts the bilirubin to excretable conjugated bilirubin. (See Web Color Image 6–4.)

2. After delivery, however, the infant’s liver cannot convert unconjugated bilirubin to conjugated bilirubin because newborns are deficient in glucuronyl transferase. This unconjugated bilirubin accumulates in the infant’s tissues, causing jaundice and brain damage (kernicterus). (See Web Color Image 6–5.)

B. Laboratory evaluation of disease. In addition to being caused by different types of antibodies, there are other differences between ABO-HDN and Rh-HDN that are identified by several laboratory tests. The hyperbilirubinemia and the degree of anemia are the primary indicators of the severity of the disease, which can range from mild to severe.

1. ABO-HDN is characterized by a weakly positive or negative DAT. Anemia is absent or very mild, but spherocytes and reticulocytes are increased. Jaundice, if present, is usually mild and does not appear for 24 to 48 hours after delivery. ABO-HDN is seen most often in group A1 or B infants who have group O mothers.

2. Rh-HDN. In contrast to ABO-HDN, Rh-HDN is characterized by a positive DAT. Anemia is present, and reticulocytes are increased. Jaundice appears within the first 24 hours, and bilirubin levels are greatly increased.

C. Prenatal testing to assess risk of HDN. If a prenatal antibody screen detects an antibody in the mother, it should be identified. The class of immunoglobulin involved is important because IgG antibodies cross the placenta, but IgM antibodies do not. In the case of antibodies that have both an IgG and an IgM component, DTT can be used to eliminate the agglutinating capability of the IgM portion, which allows the IgG component to be titered.

D. Fetomaternal bleeds, and thus the risk of sensitization, can be assessed by:

1. A qualitative test, such as the rosetting test, distinguishes Rh-positive fetal RBCs from Rh-negative maternal RBCs. Anti-D is added to maternal RBCs. Any Rh-positive cells present will attach to the anti-D. D-positive test cells are then added and form rosettes with any anti-D coated fetal cells present.

2. A quantitative test, such as the Kleihauer-Betke stain, distinguishes hemoglobin F-containing fetal RBCs from those adult cells that contain hemoglobin A. An alcohol-fixed blood smear is treated with an acid buffer to elute the hemoglobin A. The cells are then counterstained. Hemoglobin F-containing cells stain, and the hemoglobin A-containing cells appear as ghost cells.

E. Treatment of HDN can include:

1. Intrauterine transfusion to correct anemia in the fetus
2. Early delivery, usually at or after 34 weeks’ gestation, and when fetal lung maturity has been determined
3. Transfusion to correct anemia in the newborn in mild cases
4. Exchange transfusion in severe cases:
   a. To decrease bilirubin levels
   b. To correct anemia
   c. To remove the infant’s sensitized RBCs
   d. To decrease the concentration of incompatible antibodies
5. Donor blood cell characteristics
   a. Must be group-specific or must be negative for the antigen against which the
      mother’s antibodies are directed
   b. Should be group O or the same ABO group as the mother and infant, if both are
      the same
   c. Must not have any unexpected antibodies
   d. Must be less than 7 days old
   e. Should be negative for CMV
   f. Should be negative for hemoglobin S

F. Prevention of HDN
1. The only type of HDN now preventable is that caused by anti-D. The administration of
   Rh immune globulin (RhIG) within 72 hours after delivery of the first D-positive infant
   from a D-negative mother prevents the sensitization of the mother to subsequent D-
   positive fetuses. D-negative or D* negative patients can be given RhIG at approximately
   28 weeks’ gestation. RhIG is made of purified, concentrated anti-D gamma globulin.
   (See Web Color Image 6–6.)
2. Criteria for administering RhIG
   a. Mother must be D-negative; D-negative, Du positive patients are not usually given
      RhIG
   b. Mother must have no detectable anti-D in her serum
   c. Infant must be D-positive
   d. The DAT on the cord blood must be negative
   e. The amount of RhIG to be given is calculated by performing a Kleihauer-Betke
      test and using the following formula:
      \[
      \text{Number of fetal cells} \times \frac{5000}{1000 \text{ Adult cells}} = \frac{\text{Number of vials to give}}{30}
      \]
      One vial of RhIG should be given if no fetal cells are detected.

XI. AUTOIMMUNE HEMOLYTIC ANEMIAS (AIHA). Autoantibodies
may be responsible for decreased RBC survival and may interfere with pretransfusion
testing. There are several categories of AIHA (Table 6–13).

A. Warm AIHAs account for the majority of cases of AIHA. Antibodies involved are usually
IgG and react best at 37°C. The DAT is almost always positive. If the antibody screen is
positive, adsorption and elution studies may be necessary to determine if the antibodies
present are autoantibodies, alloantibodies, or both.

B. Cold AIHAs are the second most common type of AIHA. These are usually IgM antibod-
ies and may be harmless or cause disease, such as cold agglutinin disease. PCH is often

<table>
<thead>
<tr>
<th>AIHA Type</th>
<th>DAT Reaction Temperature (°C)</th>
<th>DAT</th>
<th>Reactive Eluate</th>
<th>Antibody in Serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Warm</td>
<td>&gt;37</td>
<td>+</td>
<td>+</td>
<td>yes</td>
</tr>
<tr>
<td>Cold</td>
<td>&lt;37</td>
<td>0</td>
<td>+</td>
<td>no</td>
</tr>
<tr>
<td>Mixed</td>
<td>&lt;37, &gt;37</td>
<td>+</td>
<td>+</td>
<td>yes</td>
</tr>
<tr>
<td>PCH</td>
<td>≤37</td>
<td>0</td>
<td>+</td>
<td>no</td>
</tr>
<tr>
<td>Drug-induced Adsorption</td>
<td>37</td>
<td>0</td>
<td>+</td>
<td>yes</td>
</tr>
<tr>
<td>Immune complex</td>
<td>37</td>
<td>0</td>
<td>+</td>
<td>no</td>
</tr>
<tr>
<td>Membrane modification</td>
<td>37</td>
<td>+</td>
<td>+</td>
<td>no</td>
</tr>
<tr>
<td>Drug-induced</td>
<td>37</td>
<td>+</td>
<td>0</td>
<td>yes</td>
</tr>
</tbody>
</table>

DAT = direct antiglobulin test; PCH = paroxysmal cold hemoglobinuria
Table 6–14: Drugs Capable of Causing Autoimmune Hemolytic Anemias (AIHA)

<table>
<thead>
<tr>
<th>Mechanism of AIHA</th>
<th>Drug</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drug adsorption</td>
<td>Penicillin</td>
</tr>
<tr>
<td></td>
<td>Cephalothin</td>
</tr>
<tr>
<td></td>
<td>Quinidine</td>
</tr>
<tr>
<td>Immune complex</td>
<td>Rifampin</td>
</tr>
<tr>
<td></td>
<td>Phenacetin</td>
</tr>
<tr>
<td></td>
<td>Quinine</td>
</tr>
<tr>
<td>Membrane modification</td>
<td>Cephalothin</td>
</tr>
<tr>
<td>Autoantibody</td>
<td>Methyldopa</td>
</tr>
<tr>
<td></td>
<td>Levodopa</td>
</tr>
<tr>
<td></td>
<td>Mefenamic acid</td>
</tr>
</tbody>
</table>

associated with transient infections. The antibody involved is auto-anti-P (the Donath-Landsteiner antibody). Pathologic autoantibodies bind to RBCs at temperatures between 30°C and 32°C. Hemolysis occurs when the temperature increases to 37°C. Such antibodies are referred to as biphasic. The DAT is positive because of the presence of C3d on the RBCs. Cold-reacting antibodies may interfere with pretransfusion testing, and adsorption studies may be helpful in determining if alloantibodies are present.

C. Mixed-type AIHAs cause severe hemolysis. These AIHAs have features of both warm and cold AIHAs. The DAT is positive because of the presence of both IgG and C3d on the RBCs. Adsorption studies may be necessary in pretransfusion testing.

D. Drug-induced immune hemolytic anemias will give a positive DAT. Table 6–14 lists some drugs known to cause AIHAs. Four different mechanisms of drug-induced hemolysis have been identified:

1. Drug adsorption. The involved antibodies are IgG and are directed against drugs such as penicillin that are adsorbed onto the surface of the RBCs. Eluates react only with drug-sensitized RBCs.
2. Immune complex type. Antibodies to drugs such as quinine and quinidine form immune complexes with the drugs and activate complement. C3d is usually found on the RBCs. Eluates are nonreactive.
3. Membrane modification. Drugs such as cephalothin modify the RBC membrane and allow the nonspecific adsorption of protein. Eluates are nonreactive.
4. Drug-induced hemolytic anemias. The DAT is usually positive because of the presence of IgG on the RBCs and becomes positive within 3 to 6 months after starting therapy with procainamide, levodopa, methyldopa, or mefenamic acid. A positive DAT may persist for up to 2 years after the particular drug has been discontinued. Eluates in this type of AIHA are reactive with normal RBCs.

XII. TRANSFUSION-TRANSMITTED DISEASES. Many different infectious diseases can be transmitted through transfused blood and blood products. Although the careful testing of donor units has significantly decreased the incidence of such diseases, the risk of acquiring them through transfusion still exists. These diseases include:

A. Hepatitis
   1. Hepatitis A infection through blood transfusion is extremely rare.
   2. The incidence of hepatitis B infection through blood transfusion has been greatly reduced by mandatory testing of all donor units for HBsAg.
   3. Hepatitis C infection was the cause of 90% of all cases of non-A, non-B post-transfusion hepatitis. Recently developed testing methods have reduced the risk of transfusion-transmitted infection. All donor units must be tested for hepatitis C.
   4. Hepatitis D infection is seen only in conjunction with hepatitis B infections.
B. HIV. HIV-1 and HIV-2 are known to cause disease in humans, although HIV-2 infection is currently extremely rare in the United States. Testing for HIV-1 and HIV-2 is required on all donor units. Improved testing techniques have greatly reduced the risk of transfusion-transmitted HIV infections.

C. HTLV-I and HTLV-II antibodies have been identified in some intravenous drug users. All donor units are tested for both viruses, and transfusion-related transmission is rare.

D. CMV transmission is of concern in low-birth-weight neonates and immunocompromised patients. Because the virus is transmitted by leukocytes, the transfusion of leukocyte-depleted donor units is helpful in reducing the risk of infection.

E. Malaria. Risk of transfusion-transmitted malarial infections is extremely rare. There are no screening tests, but persons with a history of disease are temporarily excluded from blood donation.

F. Babesiosis. Risk of transfusion-transmitted infection by Babesia microti is extremely rare. There are no screening tests.

G. Syphilis is rarely transmitted through blood transfusion, because the period that viable spirochetes can be found in the blood is very brief. The FDA requires syphilis testing on all donor units.

XIII. THE MAJOR HISTOCOMPATIBILITY COMPLEX (MHC) AND HUMAN LEUKOCYTE ANTIGENS (HLA)

A. The MHC is a set of closely linked genes found in all vertebrates that are capable of mounting an immune response. The cell-surface antigens that are encoded for by these genes play a major role in both immunity and disease. There are three classes of MHC proteins (see Section XIII B 1–3):

1. Class I MHC proteins
2. Class II MHC proteins
3. Class III MHC proteins

B. HLA. The MHC in humans is called the HLA complex. The antigens in this system are identified as HLA-A, -B, -C, -DP, -DQ, or -DR, all of which can be classified as class I, II, or III.

1. Class I HLA antigens are HLA-A, HLA-B, and HLA-C proteins. They are found on all tissue cells in the body as well as on platelets. They are present only in very small amounts on RBCs. These RBC HLA antigens are the Bg antigens, which include HLA-B7, HLA-B17, and HLA-A28.

2. Class II HLA antigens are HLA-DR, HLA-DQ, and HLA-DP. These antigens are capable of inducing a humoral immune response. They are expressed only on B lymphocytes and antigen-presenting cells, such as monocytes, macrophages, dendritic cells, Langerhans cells, and Kupffer’s cells. All cells that express class II MHC proteins also express class I proteins.

3. Class III HLA antigens include the complement precursors C2 and C4, as well as the Chido and Rodgers RBC antigens.

C. HLA and disease. Many diseases, including a significant number of autoimmune diseases, have been associated with specific class II HLA antigens. Determining an individual’s HLA type can help predict the risk of certain diseases. Table 6–15 lists some diseases and the HLA antigens associated with an increased risk of developing these diseases.

D. Organ transplantation. The cellular and humoral immune responses that can result in organ-graft rejection are caused by HLA antigens. Testing of both donor and recipient for
Table 6–15  Selected Human Leukocyte Antigens (HLA) and Associated Diseases

<table>
<thead>
<tr>
<th>HLA Antigen</th>
<th>Associated Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLA-B7</td>
<td>Multiple sclerosis</td>
</tr>
<tr>
<td>HLA-B8</td>
<td>Myasthenia gravis</td>
</tr>
<tr>
<td></td>
<td>Systemic lupus erythematosus</td>
</tr>
<tr>
<td></td>
<td>Graves’ disease</td>
</tr>
<tr>
<td></td>
<td>Addison’s disease</td>
</tr>
<tr>
<td>HLA-B27</td>
<td>Ankylosing spondylitis</td>
</tr>
<tr>
<td></td>
<td>Juvenile rheumatoid arthritis</td>
</tr>
<tr>
<td></td>
<td>Reiter’s syndrome</td>
</tr>
<tr>
<td>HLA-DR2</td>
<td>Goodpasture’s syndrome</td>
</tr>
<tr>
<td></td>
<td>Narcolepsy</td>
</tr>
<tr>
<td>HLA-DR3</td>
<td>Juvenile diabetes mellitus</td>
</tr>
<tr>
<td>HLA-DR4</td>
<td>Rheumatoid arthritis</td>
</tr>
<tr>
<td></td>
<td>Juvenile diabetes mellitus</td>
</tr>
</tbody>
</table>

HLA compatibility greatly reduces the risk of rejection. HLA-A, HLA-B, and HLA-DR antigens are the most important antigens in compatibility testing for solid organ transplants.

E. Bone marrow transplantation can result in severe GVHD and graft rejection. Donor marrow that matches the recipient’s HLA-A, HLA-B, HLA-C, and HLA-DR antigens is less likely to stimulate rejection.

F. Paternity testing. HLA-A and HLA-B typing in combination with RBC antigen testing can exclude an individual as a father in 95% of cases. There are two types of exclusions.

1. Direct or first-order exclusion is established when a child inherits a trait that neither the alleged mother nor the alleged father has.
2. Indirect or second-order exclusion is established when:
   a. The alleged father is homozygous for an antigen that the child does not have.
   b. The child is homozygous for an antigen that the mother has but the alleged father does not have.
   c. The child does not have two antithetical antigens that the alleged father has.

XIV. ALTERNATIVE METHODOLOGIES IN BLOOD BANK TESTING

A. Gel Technique. The antibody screening may also be performed using a microtubule filled with dextran acrylamide gel. The screening cells used for this technique meet the same criteria as for the tube test but are suspended in LISS to a concentration of 0.8%.

1. Add 50 mL of 0.8% RBCs to microtubes
2. Add 25 mL of patient serum to RBCs
3. Incubate for 15 minutes at 37°C (sensitization stage)
4. Centrifuge for 10 minutes (RBCs are forced out of the reaction chamber down into the gel which contains anti-IgG.) If sensitization occurred, the anti-IgG will react with the antibody-coated cells, resulting in agglutination, which will be trapped within the gel
5. Evaluate reactions and record results.
6. Entraps RBCs and facilitates a stabilized serologic reaction

B. Solid phase technique. The target antigen on the RBCs is affixed to the bottom of the microtiter wells. The test plasma or serum and LISS are added to the wells, and then are incubated at 37°C to allow time for possible antibodies to attach to the antigen in the well. The wells are then washed to remove unbound plasma or serum. Indicator cells (AHG-coated RBCs) are then added and the microplates are centrifuged. If antibody has attached to the antigen, the indicator cells will form a monolayer of RBCs. If no antibody is present,
nothing is attached to the antigen, and the indicator cells form a clearly delineated button at the center of the microplate well following certification.
CHAPTER 7
Clinical Microbiology

LYNNE HAMILTON, PhD, MT (ASCP) AND HAL LARSEN, MT (ASCP), CLS (NCA), PhD

I. BACTERIA

A. Prokaryotic organisms
1. Bacteria are unicellular organisms that lack a true nucleus and nuclear membrane. The lack of mitochondria, endoplasmic reticulum, and Golgi bodies also differentiates prokaryotic bacteria from eukaryotes.
2. The bacterial genome is a single, closed, circular chromosome of double-stranded DNA called the nucleoid. Plasmids are small circular molecules of extrachromosomal circular DNA. Antibiotic resistance is often encoded by genes located on plasmids. Chromosomal or plasmid gene exchange via transformation, transduction, or conjugation are means of genetic recombination.
3. Reproduction of bacteria is achieved by binary fission, a means of asexual reproduction. Prokaryotes do not reproduce sexually.
4. Energy generation of bacteria is cytoplasmic membrane-associated via the electron transport chain.
5. Bacteria usually range in size from 0.2 to 2 um in diameter and 1 to 6 um in length. The four basic morphological types are cocci (spherical-shaped cells), bacilli (rod-shaped cells), spirilla (spiral-shaped cells), and vibrios (comma-shaped cells (Figure 7–1).

B. Growth and nutrition
1. Prokaryotic bacteria have three major nutritional needs for growth.
   a. A source of carbon is needed for the synthesis of cellular constituents.
   b. A source of nitrogen is necessary for the synthesis of protein.
   c. Energy (ATP) is needed for cellular functions.
2. The optimum pH for the growth of most bacteria is 7.0 to 7.5. Acidophiles grow at an acidic pH and alkaliphiles grow optimally at an alkaline pH.
3. A wide range of growth temperatures facilitate the growth of bacteria. The optimum growth temperature for most human bacterial pathogens is 35°C to 37°C, the temperature of the human body.
4. Bacteria may be classified based on the atmospheric requirement for growth.
   a. Obligate aerobes require oxygen for growth.
   b. Aerotolerant aerobes can grow in the presence of oxygen, but grow best in an anaerobic environment.
   c. Facultative anaerobes grow in both aerobic and anaerobic environments.
   d. Obligate anaerobes cannot grow in the presence of oxygen.
   e. Capnophiles bacteria require concentrations of 5% to 10% CO2, for optimal growth.
   f. Microaerophilic bacteria grow optimally in a reduced level of oxygen.
C. Metabolism

1. Fermentation is an anaerobic process by which bacteria catabolize carbohydrates to produce energy. The process is carried out by both obligate and facultative anaerobes, and the final electron acceptor is an organic compound.

2. Respiration (oxidation) is an efficient process by which obligate aerobes and facultative anaerobes generate energy. Molecular oxygen is the final electron acceptor. Respiration is a more efficient mechanism for energy generation than fermentation.

II. BACTERIAL CELL STRUCTURE

A. The cell membrane serves as an osmotic barrier and may be a site of antibiotic action. An intact membrane is essential for bacterial viability.

B. The cell wall

1. The most prominent layer of the gram-positive cell wall is the thick, rigid peptidoglycan layer, the site of action of the penicillins and cephalosporins. Teichoic and lipoteichoic acids are unique to the gram-positive cell wall (Figure 7–2).

2. More complex than the gram-positive cell wall, the gram-negative cell wall contains a thinner peptidoglycan layer, but also an outer lipopolysaccharide (LPS) layer. LPS is an endotoxin that is an important virulence factor. An endotoxin causes shock, sepsis, fever, disseminated intravascular coagulation (DIC), and leukopenia (Figure 7–3).

C. A polysaccharide capsule covers many bacteria and serves to prevent or inhibit phagocytosis. For many organisms (e.g., Streptococcus pneumoniae), the capsule is the chief determinant of virulence.
D. Pili, also called fimbriae, are short, hairlike structures that serve to attach bacteria to target cells. For many bacteria (e.g., Neisseria gonorrhoeae), interference with attachment prevents infection. The exchange of deoxyribonucleic acid (DNA) between bacteria during conjugation occurs through the pili.

E. Flagella determine motility and can be used in classification.

F. Endospores are a means of survival that make disease control very difficult. The two spore-forming genera of importance are Bacillus and Clostridium.
III. STAINS

A. Gram’s stain
1. Crystal violet is the primary stain. Iodine binds the crystal violet to the cell wall (mordant). Decolorizer washes out any unbound dye. Safranin O is a counterstain.
   a. Gram-positive cells retain the crystal violet and stain purple (see Web Color Images 7–1, 7–2, and 7–3).
   b. Gram-negative cells are decolorized, retain the safranin O counterstain, and stain red or pink (see Web Color Images 7–4 and 7–5).
2. Clinical use. Gram’s stain is especially useful for examining smears of clinical specimens. Initial treatment, and often a presumptive identification, can be made from Gram’s stain results. White and red blood cells (WBCs and RBCs), as well as cellular debris, stain pink. This can serve as an internal control.

B. Acridine orange stain is an orange fluorescent stain used to detect bacteria in body fluids in which numbers of bacteria may be few (e.g., blood and spinal fluid). The stain can also be used to detect bacteria in direct smears with excess cellular debris. It is very sensitive and can detect small numbers of bacteria that are living or dead. The procedure consists of flooding a methanol-fixed smear with acridine orange for 2 minutes, washing, and then observing with a fluorescence microscope (see Web Color Image 7–6).

C. Methylene blue stain is especially helpful for demonstrating metachromatic granules and characteristic morphology of Corynebacterium diphtheriae from Loeffler coagulated serum medium. The procedure consists of flooding a fixed smear with methylene blue, followed by washing (see Web Color Image 7–7).

D. Acid-fast stain is used to detect organisms that do not stain well with other conventional stains (e.g., Mycobacterium spp., Nocardia, Actinomyces). These organisms have a high lipid content in their cell walls. Once stained, they are very resistant to decolorization by acid alcohol.
   The most commonly used method is a carbolfuchsin stain, the modified Kinyoun stain. The primary stain is carbolfuchsin, which contains a surface-active detergent to facilitate penetration of the stain without heating. After washing, methylene blue is used as a counterstain. Acid-fast organisms appear red against a blue background. The Ziehl-Neelsen is a carbolfuchsin stain that utilizes heat to drive the stain into the mycobacterial cell wall (see Web Color Image 7–8).

E. Auramine-rhodamine stain is a fluorescent stain that detects mycolic acids and can be used for staining acid-fast organisms. The smear is stained with auramine-rhodamine, decolorized with acid alcohol, and then flooded with potassium permanganate. It is observed with a fluorescence microscope. The cells appear yellow against a dark background (see Web Color Image 7–9).

F. Calcofluor white stain binds specifically to chitin, which is found in fungal cell walls. It is used to detect yeast cells and hyphae in skin scrapings and other specimens. The fungal elements appear green or blue-white (see Web Color Image 7–10).

IV. NORMAL FLORA describes the microorganisms that are frequently found on or in the bodies of healthy persons.

A. General characteristics
1. Local conditions select for those organisms that are suited for growth in a particular area.
2. Resident flora colonize an area for months or years.
3. Transient flora are present at a site temporarily.
4. Organisms that live at the expense of the host are parasites.
5. Organisms that benefit the host are symbionts.
6. Commensals have a neutral effect on the host.
7. A carrier harbors the organism without manifesting symptoms, but is capable of transmitting infection (carrier state).
8. Opportunists are organisms that do not normally cause infection, but can do so if the condition of the host changes (e.g., immunosuppression).

B. Normal flora of the skin
1. The skin contains a wide variety of microorganisms that are not eliminated by washing or superficial antisepsis.
   a. Propionibacterium acnes colonizes the sebaceous glands.
   b. Micrococcus, Staphylococcus spp., and diphtheroids are common.
2. Intact skin is an effective barrier to microbial invasion.

C. Normal flora of the mouth. The mouth contains large numbers of bacteria, most commonly Streptococcus spp. (especially viridans species), coagulase-negative Staphylococcus, Peptostreptococcus, and other anaerobes.

D. Normal flora of the respiratory tract
1. The respiratory tract beyond the oropharynx is normally sterile.
2. The ciliary action of epithelial cells and mucus movement remove invading organisms.
3. The nose and nasopharynx contain Staphylococcus aureus, S. epidermidis, and Streptococcus spp. The following may be present transiently during community outbreaks of infection: Streptococcus pneumoniae, Haemophilus influenzae, and Neisseria meningitidis.
4. The normal flora in the oropharynx mirrors that of the mouth.

E. Normal flora of the gastrointestinal tract
1. Most microorganisms are destroyed in the stomach due to the acidic pH. The survivors multiply in the colon.
2. More than 90% of the microbial population is comprised of anaerobes.
3. Alteration of the normal flora by antibiotics may allow a superinfection by Clostridium difficile (necrotizing enterocolitis), Candida albicans, or S. aureus.
4. The most commonly found organisms in the gastrointestinal tract are: Bacteroides spp., Clostridium spp., Eubacterium spp., anaerobic streptococci, Enterococcus spp., and Enterobacteriaceae.

F. Normal flora of the genitourinary tract
1. The outermost segment of the urethra is colonized by skin organisms.
2. The vagina is colonized with Lactobacillus, anaerobic gram-negative rod-shaped bacteria, and gram-positive cocci.

V. PATHOGENESIS OF INFECTION

A. Host resistance
1. Innate immunity, or natural immunity, is a nonspecific mechanism of resistance to disease.
   a. Physical barriers such as the skin, mucous membranes, and cilia help prevent the invasion of pathogenic bacteria.
   b. The cleansing action of fluids in the eyes, respiratory, digestive, urinary, and genital tracts are also effective host resistance mechanisms.
   c. Antimicrobial substances such as secretory IgA, lysozyme, B-lysin, and interferon are produced in the human host.
   d. Indigenous microbial flora compete with invading bacteria for colonization sites, as well as produce inhibitory bacteriocins.
e. Phagocytosis, inflammation, and complement activation are also important non-specific defense mechanisms of the innate immunity.

2. Acquired or specific immunity develops in response to a specific foreign antigen (e.g., invading bacterium). Acquired immunity may be active (a response to immunization or disease) or passive (through placental transfer or infusions of serum or plasma).
   a. The humoral response involves antibody production by B lymphocytes.
   b. Cell-mediated immunity involves cells, such as macrophages, cytotoxins, and T lymphocytes. The cellular response provides immunity against intracellular pathogens, including viruses, fungi, and mycobacteria.

B. Criteria for a successful pathogen
   1. The degree of pathogenicity of a microorganism is directly related to its virulence. A virulent strain is characterized by its ability to evade or overcome host defenses and cause disease. Microbial virulence factors include adhesions, capsular polysaccharides, and production of extracellular toxins and enzymes that may cause damage to the host.
   2. An appropriate portal of entry is necessary for successful transmission of disease. For example, respiratory pathogens enter the respiratory tract via inhalation of aerosolized respiratory secretions. Gastrointestinal pathogens enter the GI tract via ingestion of contaminated food and water.
   3. A sufficient infective dose is needed for a microorganism to be able to cause disease in its host. Some organisms can establish an infection with a relatively low infective dose (e.g., Shigella), whereas others require a much higher infective dose to cause infection (e.g., Salmonella).
   4. A successful pathogen must be able to overcome the host resistance mechanisms, including the immune responses, and cause disease in a susceptible host.
   5. For continued transmission of disease, pathogenic microorganisms must be able to leave the host via an appropriate portal of exit. For most pathogens, it is the same as the portal of entry.

C. Transmission
   1. Airborne transmission involves the respiratory spread of infectious disease via aerosolized respiratory secretions or contact with contaminated inanimate objects or fingers and hands.
   2. Ingestion of contaminated food and water is the usual means for transmission of gastrointestinal infections. Infection can also occur via the fecal-oral route.
   3. Sexual transmission is the route of infection for venereal diseases. Some diseases can be also transmitted via skin-to-skin contact or via direct transfer of saliva (e.g., kissing).
   4. Animal and human bites can result in serious wound infections, usually caused by mouth flora. Rabies is an example of a viral infection transmitted from an animal bite. Opportunistic pathogens, including environmental bacteria, can enter the human via cuts and trauma wounds.
   5. Arthropod vectors, such as ticks, fleas, and mites, are responsible for the transmission of diseases such as malaria, Lyme disease, plague, Rocky Mountain spotted fever, and hemorrhagic fevers. The infectious agent multiplies in the arthropod and is transmitted when the arthropod feeds off a human host.
   6. Zoonoses are diseases of animals that are transmitted to humans. Transmission is usually via contact with infected animals, animal secretions, or animal products. Zoonoses can also be transmitted via animal bites or arthropod vectors.

VI. COLLECTION AND HANDLING OF CLINICAL SPECIMENS
A. Collection. A properly collected specimen is absolutely crucial to quality diagnostic information and patient care.
   1. Safety
a. **Universal precautions** are followed throughout the collection and handling process. Persons collecting or handling specimens should wear gloves and a laboratory coat. Eye protection should also be worn if splashing is a potential hazard.

b. Accidents or injuries must be reported immediately.

2. **General guidelines**
   a. The specimen should be from the infection site and not contaminated by the surrounding area (e.g., culture within a wound and not the surface or the surrounding skin).
   b. Whenever possible, the specimen should be collected before antimicrobials are administered.
   c. Appropriate collection devices and containers should be used and must be sterile. Aseptic technique is required.
   d. The specimen container should be labeled with the patient’s identification, the date and time of collection, and the source of specimen.

3. **Collection from various body sites**
   a. **Throat.** The tongue should be depressed before swabbing between the tonsillar pillars and behind the uvula. The cheek, tongue, and teeth should not be touched.
   b. **Nasopharynx.** A flexible wire nasopharyngeal swab should be gently inserted through the nose into the posterior nasopharynx, rotated, and then removed.
   c. **Sputum.** Whenever possible, the patient should gargle with water (not mouthwash) immediately before sampling. Early morning specimens are best. Expectorated specimens from a deep cough should be collected into a sterile specimen cup.
   d. **Stool.** Specimens should be collected in a clean, wide-mouthed container with a tight-fitting lid. If the specimen cannot be plated within 1 hour of collection, it should be mixed with a transport medium (e.g., buffered glycerol saline, Cary-Blair transport medium). The change in pH and temperature over time is detrimental to *Shigella* spp. Stool specimens should never be taken from the toilet and should not be contaminated with urine. Commercial systems with preservatives are available for collection of specimens for both bacterial culture and ova and parasite examination.
   e. **Urine.** Midstream clean-catch is the most common collection method. Proper cleansing of the urethral area is important, especially in women. The first few milliliters, which flush out the urethra, are discarded. Proper collection of urine specimens usually have less contaminating bacterial flora.
   f. **Blood.** Two to three cultures should be collected at random times during a 24-hour period. Collecting more than three sets of cultures in a 24-hour period does not significantly increase the probability of detecting bacteremia. Skin is disinfected with 70% alcohol, followed by iodine. The disinfectant is allowed to dry. The puncture site should not be palpated after disinfection. Ideally, 20 to 30 mL of blood per culture is collected from an adult (1–5 mL from infants and small children). Iodine should be cleaned from the puncture site with alcohol following the venipuncture.
   g. **Cerebrospinal fluid** should be collected aseptically by a physician. This specimen should be processed immediately and not exposed to heat or refrigeration.
   h. **Abscess aspirates or exudates, as well as synovial, pericardial, and chest fluid** should be collected by a physician with a needle and syringe. The use of swabs may inhibit growth of anaerobes or increase the likelihood of contamination with indigenous bacteria from adjacent tissues (e.g., mucous membranes or superficial skin surfaces). Care should be taken not to inject an air bubble into the syringe.
   i. **Genital tract**
      1. **Men.** Exudates may be expressed from the urethral orifice or a small-diameter swab may be inserted 3 to 4 cm into the urethra. The specimen should be plated immediately on the appropriate media and not allowed to dry or be exposed to cold temperatures. A direct Gram’s stain smear should be prepared.
      2. **Women.** Cervical specimens are obtained by a physician with the aid of a speculum. Lubricants, which may be lethal to *Neisseria gonorrhoeae*, should
not be used on the speculum. The cervical mucus plug is removed, and a sterile swab is inserted into the cervix, rotated, and allowed to remain for a few seconds. The specimen should be plated immediately to the appropriate media, and swabs should not be refrigerated, as refrigeration may be lethal to genital pathogens. A smear for Gram’s stain should be prepared from the specimen.

B. Handling

1. Transport all specimens to the laboratory promptly. Anaerobic specimens must be transported in an anaerobic transport system. If transport cannot occur immediately, most specimens can be held at 2°C to 8°C. Exceptions to this include specimens that likely contain temperature-sensitive organisms (e.g., Neisseria), blood culture bottles, and cerebrospinal fluid (CSF). Generally, swabs are the least desirable collection and transport method. However, organisms can be successfully cultured if the swab is handled and transported properly (i.e., not allowed to dry out). Swabs are inappropriate for culturing anaerobes, although in some clinical settings, culturettes are often used. If swabs are used for the culture of anaerobes, an anaerobic culturette must be used. Use of aerobic culturettes for the culture of anaerobes is criteria for specimen rejection.

2. Processing

a. Media selection. Some general principles apply to the use of primary plating media. In most cases, the concern is that the primary media will grow and isolate all or a majority of the possible organisms from a clinical specimen. In those cases in which certain organisms are excluded, the decision of what media is used is based on time, cost, and probability of isolation information. In many cases, the choice of primary media is an individual laboratory choice. Selective and differential media may be used in addition to all-purpose and enriched agars. These specialized media are used for selective recovery and preliminary differentiation of specific bacteria. It is especially important that the microbiologist understands the range and purpose of each primary isolation medium, as well as the various reactions of the organisms, since individual organism groups may react differently on specific media.

(1) Most isolation protocols call for the use of blood agar (with 5% sheep RBC).
(2) Chocolate agar is used for fastidious isolates.
(3) Specialized media [e.g., mannitol salt agar, bismuth sulfite agar, Campylobacter agar, thiosulfate-citrate-bile salts-sucrose (TCBS) agar] are used when specific organisms are suspected.
(4) Substitutions may be made with acceptable results (e.g., MacConkey agar in place of Eosin Methylene Blue agar).
(5) Prereduced anaerobically sterilized (PRAS) culture media is recommended for the culture of anaerobes (see anaerobe section).

b. Incubation

(1) The normal incubation temperature for bacterial cultures is 35°C to 37°C. Culture plates may be incubated in ambient air, but incubation in a capnophilic atmosphere of 5% to 10% CO2 is recommended to enhance the growth of fastidious bacterial isolates. Anaerobic cultures should be incubated anaerobically at 35°C to 37°C.
(2) Anaerobic bags, jars, or an anaerobic chamber are appropriate for incubation of anaerobic cultures.
(3) Recommended incubation of stool cultures for isolation of Campylobacter jejuni is in a microaerophilic, capnophilic atmosphere at 42°C to 45°C.

c. Specimen rejection criteria. Rejection criteria should be part of the written policy of every clinical laboratory. These criteria should be clearly listed and made available to anyone who might submit specimens for culture. Processing and culture of inappropriate specimens leads to increased costs and misinformation. In the event a specimen is rejected, the person submitting the request should be contacted and informed. In some cases, the difficulty of collection makes culturing necessary, although the results are not optimal. The following situations or
specimen types should be rejected; however, this is not intended to be a comprehensive list:

1. Twenty-four-hour urine or sputum collections
2. Specimens received in nonsterile or contaminated containers (including those in which the specimen has leaked out)
3. Specimens contaminated with barium or other foreign substances
4. Culturing of Foley catheter tips
5. Saliva instead of sputum
6. Unrefrigerated urine specimens 2 hours or more post-collection
7. Anaerobic culturing of midstream urine, upper respiratory tract, superficial skin, or feces specimens (certain Clostridium species may be appropriately cultured from feces)

VII. MICROCOCCACEAE

A. General characteristics
1. Members of the family Micrococcaceae are gram-positive cocci (see Web Color Image 7–1), aerobic or facultative anaerobes, and catalase-positive (except Stomatococcus). The catalase test differentiates the Micrococcaceae from the gram-positive, catalase-negative Streptococcaceae.
2. Hydrogen peroxide is converted to water and oxygen in the presence of the bacterial enzyme catalase. The observation of vigorous bubbling when the bacterium is mixed with a drop of 3% hydrogen peroxide is a positive test (see Web Color Image 7–11).
3. Most are members of the indigenous flora and are commonly isolated from a wide variety of diseases.
4. Staphylococcus (Table 7–1; Figure 7–4) a. The staphylococci are catalase-positive, nonmotile, facultative anaerobes that are normal inhabitants of the skin and mucous membranes. These organisms commonly cause human infections.
   b. Species are initially differentiated by the coagulase test (see Web Color Images 7–12 and 7–13). The most important coagulase-positive species is S. aureus. Some animal species produce coagulase, but are rarely isolated from human samples.
   c. Staphylococci that do not produce coagulase are called “coagulase-negative staphylococci.” The most prominent species are S. epidermidis and S. saprophyticus.
5. Micrococcus (Table 7–1; Figure 7–4). Micrococci are opportunistic pathogens found only in immunocompromised persons. Micrococcus is of low pathogenic significance, but may be isolated as a contaminant or as part of the normal flora.
6. Stomatococcus (Table 7–1; Figure 7–4). This genus is part of the normal oral flora and is rarely isolated from infection. The colonies adhere strongly to the agar surface.

B. Staphylococcus aureus
1. Infections a. Skin and wound infections caused by S. aureus are suppurative and pyogenic. Some common skin infections are boils, carbuncles, furuncles, and folliculitis.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Staphylococcus</th>
<th>Micrococcus</th>
<th>Stomatococcus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catalase</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Growth on 6.5% NaCl agar</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Modified oxidase test</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Resistance to bacitracin (0.04 μM)</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Resistance to furazolidone (100 mcg)</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

* + = positive; - = negative.
b. Food poisoning: Staphylococcal enterotoxins A and D are associated with food poisoning. The source of contamination is usually an infected food handler. Infection occurs when an individual ingests food contaminated with enterotoxin-producing strains. The heat-stable toxins are preformed in the food. Symptoms appear rapidly (2–6 hours after ingestion) and resolve within 8 to 10 hours. Symptoms may include nausea, vomiting, headache, abdominal pain, and severe cramping.

c. Scalded skin syndrome (Ritter’s disease) is an extensive exfoliative dermatitis that occurs primarily in newborns and is caused by staphylococcal exfoliative or epidermolytic toxin. It can also occur in adults, most frequently among those who have chronic renal failure or are immunocompromised. The mortality rate is low in children but high in adults.

d. Toxic shock syndrome (TSS) is a multisystem disease characterized by high fever, rash, hypotension, shock, desquamation of the hands and feet, and possible death. The etiologic agent is a strain of Staphylococcus aureus that produces enterotoxin F [toxic-shock syndrome toxin-1 (TSST-1)]. There is an association between the use of tampons and TSS, although the disease may occur in both sexes if a toxin-producing strain of S. aureus has caused infection.

e. Other staphylococcal infections
   (1) Staphylococcal pneumonia secondary to influenza can occur. The mortality rate is high.
   (2) Osteomyelitis can occur secondary to bacteremia.

f. In addition to the toxins associated with specific infections (e.g., exfoliative toxin, enterotoxins A and D, TSST-1), other toxins and enzymes are also virulence factors of S. aureus. Coagulase is the major virulence marker of the species. Protein A is a cellular component in the cell wall that helps the bacterium avoid phagocytosis. Alpha and beta hemolysins cause the hemolysis of RBCs. In addition, hyaluronidase prevents the spread of infection and lipase facilitates colonization of the bacterium on the skin surface.

2. Laboratory identification of Staphylococcus aureus
   a. Microscopic examination of stained smears from clinical specimens can be especially helpful. Numerous gram-positive cocci with polymorphonuclear cells are usually seen.
b. S. aureus grows readily on common laboratory media. On sheep blood agar, colonies appear as round, smooth and white or pigmented (yellow-orange). They are usually β-hemolytic. (See Web Color Image 7–14.)
c. S. aureus is most often identified by the coagulase test. Isolates may show cell-bound (clumping factor) or free (extracellular) coagulase. Cell-bound coagulase is identified by mixing the suspected organism with a drop of rabbit plasma on a glass slide. (See Web Color Image 7–12.) If clumping occurs, the isolate demonstrates cell-bound coagulase and is identified as S. aureus. Isolates that do not clump are tested for free coagulase by the tube method (see Web Color Image 7–13), in which the organism is mixed with 0.5 mL of rabbit plasma, and following incubation at 37°C for 4 hours, is observed for clot formation.
d. Selective media that can be used to isolate S. aureus from heavily contaminated specimens or when it is the only isolate of concern are mannnitol salt agar (MSA), phenylethyl alcohol (PEA) blood agar, and Columbia-naladixic acid Agar (CNA).
   1. MSA provides mannitol as a fermentable carbohydrate source as well as 6.5% sodium chloride (NaCl). Generally, only Staphylococcus species grow on this medium because of the high salt content. S. aureus ferments the mannitol to produce acid, which turns the pink agar yellow. The colonies are identified by a yellow halo. This test is presumptive because some strains of S. epidermidis and few other species can also ferment mannitol.
   2. PEA and CNA agars both inhibit the growth of gram-negative organisms, whereas the gram-positive bacteria, including Staphylococcus species, grow well. Five-percent sheep blood is incorporated in the medium for additional enrichment and detection of hemolytic reactions.
e. Rapid methods use plasma-coated latex particles. The plasma detects clumping factor and causes agglutination of the particles. Protein A in the cell wall of S. aureus (with IgG) may also be detected with rapid methods. Other species that produce clumping factor produce positive reactions, but are tube-coagulase-negative.

C. Coagulase-negative staphylococci
   1. General characteristics. The coagulase-negative staphylococci are found as normal flora in humans and animals. The incidence of infection by these organisms has increased. They are often hospital-acquired (nosocomial). Predisposing factors include catheterization, prosthetic device implants, and immunosuppressive therapy. The most common species isolated from clinical infections are S. epidermidis and S. saprophyticus. S. saprophyticus has been associated with UTIs in young, sexually active women. Other species of coagulase-negative staphylococci are not isolated frequently. Three species that can cause a wide range of infections, but do so only occasionally, are S. haemolyticus, S. lugdunensis, and S. schleiferi. The latter two produce clumping factor and may yield a positive slide coagulase test.
   2. Laboratory identification of coagulase-negative staphylococci
      a. On sheep blood agar, colonies are usually round, smooth, and white without hemolysis. (See Web Color Image 7–15.)
      b. The most common isolates are S. epidermidis and S. saprophyticus.
      c. Urine isolates that are coagulase-negative are further tested to presumptively identify S. saprophyticus. This is done by testing for novobiocin susceptibility using a 5-mg novobiocin disk. S. saprophyticus is resistant to novobiocin, whereas other coagulase-negative staphylococci are susceptible.
      d. Species identification of the coagulase-negative staphylococci requires differentiation using many biochemical tests. Various commercial identification systems exist and may be used if appropriate.

D. Micrococcus species
   1. General characteristics. Micrococcus species are environmental organisms, as well as normal skin flora. They may also be normal flora in the respiratory tract or other sites
in the body, and are common contaminants. These coagulase-negative, gram-positive opportunists can easily be differentiated from the Staphylococcus species.

2. Laboratory identification of Micrococcus species
   a. Modified oxidase test. Modified oxidase reagent (6% tetramethylphenylene diamine hydrochloride in dimethyl sulfoxide) is added to a small amount of growth smeared onto a filter paper. Micrococci are modified oxidase-positive and turn dark blue within 2 minutes.
   b. Bacitracin susceptibility. An isolate is streaked onto a sheep blood Mueller-Hinton medium. A 0.04-U bacitracin disk is placed onto the streaked area, and the plate is incubated overnight and observed for a zone of inhibition. Micrococci are susceptible to bacitracin.
   c. Furazolidone susceptibility is tested exactly as for bacitracin susceptibility, except a disk containing 100-mcg furazolidone is used. Micrococci are resistant to furazolidone. (See Web Color Image 7–16.)

E. Antibiotic susceptibility
   1. Penicillin resistance is often so high, especially in S. aureus isolates (85% to 90%), that other antibiotics must often be used. There is variability in the susceptibility patterns.
   2. A common resistance mechanism of the staphylococci is a production of β-lactamase, an enzyme that inactivates the β-lactam antibiotics.
   3. Various β-lactamase resistant penicillins have been developed. Methicillin is the most frequently used. Oxacillin is used for in vitro susceptibility testing of methicillin resistance.
   4. Methicillin-resistant S. aureus (MRSA) and methicillin-resistant S. epidermidis (MRSE) have increased in number. These may also be referred to as oxacillin-resistant S. aureus (ORSA) and oxacillin-resistant S. epidermidis (ORSE), respectively. Vancomycin has been used as an alternative treatment of methicillin-resistant strains. However, vancomycin resistance is increasing. MRSA are etiologic agents of serious nosocomial and community-associated infections. The increased virulence of this bacterium is a major concern of physicians, epidemiologists, and the health care community.

VIII. STREPTOCOCCUS, ENTEROCOCCUS, AND RELATED GENERA

A. General characteristics
   1. The organisms included in this group are catalase-negative, gram-positive cocci (old cells may stain gram-negative or gram-variable) that are arranged in pairs or chains (see Web Color Image 7–2) and are facultative anaerobes. Growth requirements may be complex, and the use of blood or enriched medium is necessary for isolation. Their role in human disease ranges from well-established and common, to rare but increasing.
   2. Hemolysis patterns on sheep blood agar (see Web Color Images 7–17, 7–18, and 7–19) are helpful in identification (Table 7–2).

B. Streptococcus
   1. Streptococcus pneumoniae (Table 7–3)
      a. S. pneumoniae is often part of the normal flora of the respiratory tract.

<table>
<thead>
<tr>
<th>Type</th>
<th>Characteristic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alpha (α)</td>
<td>Greenish discoloration in medium surrounding colony due to partial lysis of red blood cells</td>
</tr>
<tr>
<td>Alpha-prime (α′)</td>
<td>Small ring of no hemolysis around the colony, which is surrounded by a wider zone of complete hemolysis (also called &quot;wide zone&quot; hemolysis)</td>
</tr>
<tr>
<td>Beta (β)</td>
<td>Clearing of red blood cells surrounding the colony due to complete lysis</td>
</tr>
<tr>
<td>Nonhemolytic</td>
<td>No change</td>
</tr>
</tbody>
</table>
A Concise Review of Clinical Laboratory Science

Table 7–3 Identification of Streptococcus and Related Organisms

| S. pyo- | S. agala- | Entero- | Group O | S. pneu- | Viridans | Aeroco- | Pedeco- | Leuco- |
|gococcus | genes | coccus | | moniae | streptococci | coccus | coccus | coccus |

<table>
<thead>
<tr>
<th>Hemolysis</th>
<th>β</th>
<th>β</th>
<th>a, β, non</th>
<th>a, non</th>
<th>u</th>
<th>a, non</th>
<th>u</th>
<th>u</th>
<th>u</th>
</tr>
</thead>
<tbody>
<tr>
<td>Susceptibility to:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vancomycin</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>Bacitracin</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>SXT</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>V</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>Optochin</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
</tbody>
</table>

Hippurate hydrolysis

- + - - - - - + +

PYR hydrolysis

- + - - - - - + +

CAMP test

- + - - - - - -

Bile esculin hydrolysis

- - + + m = = V + V

Growth in 6.5% NaCl

- - + - - - - + +

LAP

= + + + + + + + + + +=

b. The key virulence factor is an antiphagocytic capsule. There are approximately 80 antigenic types.

c. *S. pneumoniae* is an important human pathogen, causing pneumonia, sinusitis, otitis media, bacteremia, and meningitis. It is frequently isolated as a pathogen and as a member of the normal respiratory flora. Direct smears often reveal leukocytes and numerous gram-positive cocci in pairs. The ends of the cells are slightly pointed, giving them an oval or lancet shape. (See Web Color Image 7–20.)

de. Complex media, such as brain-heart infusion agar, trypticase soy agar with 5% sheep blood, or chocolate agar are necessary for good growth. Isolates may require increased CO₂ for growth during primary isolation. Colonies are α-hemolytic. Young cultures produce a round, glistening, wet, mucoid, dome-shaped appearance.

e. Laboratory identification. *S. pneumoniae* is susceptible to optochin (ethylhydrocuprein hydrochloride). (See Web Color Image 7–21.) The bile solubility test is also used for identification. An α-hemolytic streptococcus that is optochin-susceptible or bile-soluble can be identified as *S. pneumoniae*. Other α-hemolytic streptococci are negative for both tests. Capsular subtypes of *S. pneumoniae* are detected using the Quellung test, a microscopic “precipitin test” in which the capsules surrounding the pneumococci appear to swell. (See Web Color Image 7–22.)

2. *Streptococcus pyogenes* (Table 7–3)

a. The cell wall contains the Lancefield group A carbohydrate. This organism is also referred to as group A streptococcus or β-hemolytic group A streptococcus.

b. Virulence factors

(1) The most well-defined virulence factor is M protein. There are more than 80 different serotypes. Resistance to infection is related to the presence of type-specific antibodies to the M protein. The M protein molecule causes the streptococcal cell to resist phagocytosis. It enables the bacterial cell to adhere to mucosal cells.

(2) Streptolysin O causes hemolysis of RBCs. Its role in virulence is unknown. Antibodies to streptolysin O indicate a recent infection (antistreptolysin O titer).

(3) Hyaluronidase (spreading factor) may favor the spread of the organism through the tissues.

(4) All strains form at least one deoxyribonuclease (DNase). The most common is DNase B. These enzymes are antigenic, and antibodies to DNase can be detected following infection.
(5) Some strains of *S. pyogenes* cause a red spreading rash referred to as scarlet
fever. This condition is caused by erythrogenic toxin.
(6) Protein F is a fibronectin-binding protein that facilitates adhesion to epithelial
cells.
(7) Streptokinase causes the lysis of fibrin clots.

e. Infections

(1) Pharyngitis is one of the most common *S. pyogenes* infections. “Strep
throat” is most frequently seen in children between the ages of 5 and
15 years. Diagnosis relies on a throat culture or a positive quick “strep”
test, in which group A antigens are detected from a throat swab in a mat-
ter of minutes. A throat culture is recommended if the antigen-detecting test is
negative.
(2) Skin infections include impetigo, necrotizing fasciitis, and pyoderma.
(3) Scarlet fever is a red rash that appears on the upper chest and spreads to the
trunk and extremities following infection with *S. pyogenes*.
(4) Rheumatic fever and glomerulonephritis may result from infection at other
sites in the body. Damage appears to result from cross-reactivity of the strep-
tococcal antigens with host tissue antigens.
(5) Streptococcal TSS (toxic shock syndrome) is similar to that caused by *Staphy-
lococcus aureus*.

d. Laboratory identification. Colonies of *S. pyogenes* on blood agar are small, trans-
parent, and smooth, and they show β-hemolysis. Gram’s stain reveals gram-positive
 cocci with some short chains. The bacterium is susceptible to bacitracin or Taxo
A (see Web Color Image 7–23) and resistant to SXT. In addition, *S. pyogenes*
hydrolyzes L-pyrrolidonyl-β-naphthylamide (PYR). A positive test is develop-
ment of a red color after the addition of dimethylaminocinnamaldehyde reagent to
an inoculated PYR disk (see Web Color Image 7–24).

3. *Streptococcus agalactiae* (Table 7–3)

a. The cell wall contains the Lancefield group B carbohydrate. This organism is
also referred to as group B streptococcus. It may be found as normal flora in the
genitourinary tract.

b. Virulence factors

(1) The capsule is the most important virulence factor.
(2) Other factors (e.g., DNAase, hyaluronidase) have not been shown to be factors
in infection.

c. Infections

(1) Neonatal sepsis (usually manifest as pneumonia or meningitis) occurs soon
after birth. The most important factor in infection is the presence of group B
streptococcus in the vagina of the mother.
(2) Postpartum fever and sepsis may occur after birth and may manifest as en-
dometritis or a wound infection.

d. Laboratory identification. Group B streptococci grow on blood agar as grayish
white mucoid colonies surrounded by a small zone of β hemolysis. They are gram-
positive cocci that form short chains in clinical specimens and long chains in culture.
Group B streptococci are CAMP test-positive, demonstrating an arrowhead-
shaped area of synergistic hemolysis when streaked perpendicular to a β-hemolytic
*S. aureus* (see Web Color Image 7–25). Group B streptococci are also hippurate
hydrolysis-positive, resistant to SXT, and PYR-negative.

4. Groups C and G

a. There are three hemolytic species in Lancefield group C that are occasionally
isolated from clinical specimens: *S. equi*, *S. zooepidemicus*, and *S. equisim-
dilis*. The major species found in group G is *S. canis*. It occasionally causes in-
fection, and is part of the normal skin flora. Minute colony types of group G are
part of the *S. milleri* group, with *S. anginosus* being the most prominent
species.

b. These groups produce a variety of infections similar to those caused by groups A
and B. Group C can cause pharyngitis.
c. Laboratory identification. Groups C and G can be identified by extensive biochemical tests. However, serologic tests to identify the group carbohydrate in the cell wall of the isolate (e.g., agglutination) are best.

5. Group D (Table 7–3)
   a. The group D streptococci include \textit{S. bovis} and \textit{S. equinus}. They may be found as normal intestinal flora.
   b. The group D streptococci may be etiologic agents of bacterial endocarditis, UTIs, and other infections, such as abscesses and wound infections. An association has been made between bacteremia due to \textit{S. bovis} and the presence of gastrointestinal tumors. Isolation of \textit{S. bovis} from a blood culture may be the first indication that the patient has an occult tumor.
   c. Laboratory Identification. Hemolysis is usually absent, or \( \alpha \)-hemolysis is present. Key reactions of group D streptococci include a positive bile esculin test (formation of a black precipitate due to the hydrolysis of esculin) with no growth in 6.5% NaCl broth. Group D can be separated from \textit{Enterococcus} by the \( \beta \)-pyrrolidonyl-\( \beta \)-naphthylamide (PYR) test because it is negative and \textit{Enterococcus} is positive. (The enterococci also grow in 6.5% NaCl broth.) Serotyping should be done to identify an isolate such as \textit{S. bovis}, because it cannot be distinguished from some of the viridans group by biochemical tests alone.

6. \textit{Enterococcus} (Table 7–3)
   a. This genus is found in the intestinal tract. The species found in this genus include \textit{E. faecalis}, which is the most common isolate, \textit{E. faecium}, \textit{E. avium}, and \textit{E. durans}. These enterococcal species share a number of characteristics with the group D streptococci, including the group D antigen. They show resistance to several of the commonly used antibiotics, so differentiation from \textit{Group D Streptococcus} and susceptibility testing is important.
   b. The infections caused are similar to those caused by the group D streptococci. The most common is a urinary tract infection.
   c. Laboratory Identification. It is not difficult to differentiate between \textit{Enterococcus} and group D—streptococci. In addition to being positive for bile esculin (black precipitate), \textit{Enterococcus} species grow in 6.5% NaCl broth (see Web Color Image 7–26), are PYR-positive (see Web Color Image 7–24), and SXT-resistant.
   d. Enterococci may be screened for high-level aminoglycoside resistance because aminoglycosides are usually used in combination with ampicillin or penicillin for effective treatment of enterococcal infections. Resistant strains cannot be used for synergistic treatment. Gentamicin and streptomycin resistance can be detected with broth or agar dilution and disk diffusion tests.
   e. The emergence of vancomycin-resistant \textit{Enterococcus} (VRE), encoded by the \textit{vanA} gene, is a major concern of physicians, microbiologists, and hospital infection control personnel. \textit{E. faecalis} is the most common species, followed by \textit{E. faecalis}. Most microbiology laboratories screen for VRE colonization using vancomycin-containing agar. Susceptibility testing is performed only on clinically significant isolates.

7. Viridans streptococci (Table 7–3)
   a. The viridans group includes those \( \alpha \)-hemolytic streptococci that lack Lancefield group antigens and do not meet the criteria for \textit{S. pneumoniae}. They are part of the normal flora of the oropharynx and intestine.
   b. The most common infection caused by these organisms is subacute bacterial endocarditis.
   c. The viridans streptococci are fastidious and some strains require increased CO\(_2\) for growth. Identification of the viridans streptococci to the species level is a difficult task. Part of the reason for this is that there is not widespread agreement on a classification scheme. \textit{Species} of viridans streptococci include \textit{S. mutans}, \textit{S. salivarius}, \textit{S. sanguis}, \textit{S. mitis}, and \textit{S. milleri} (not \( \beta \) hemolytic).

8. Nutritionally variant streptococci (NVS)
   a. The NVS subgroup of viridans streptococci are nutritionally deficient and have been isolated from patients who have endocarditis and otitis media. This
subgroup is also known as pyridoxal (vitamin B6)-dependent, thiol-dependent, or symbiotic streptococci. Pyridoxal is not present in most liquid and solid bacteriologic media, so bacteriologic media must be supplemented with pyridoxal (vitamin B6) to support the growth of NVS. The NVS colonies are small, measuring 0.2 to 0.5 mm in diameter. When gram stained, the morphology can vary from classic gram-positive streptococci to gram-negative or gram-variable pleomorphic forms. As the optimal concentrations or required nutrients decrease, the cells become pleomorphic, even showing globular and filamentous forms.

b. NVS satellite around or grow adjacent to staphylococcal isolates. The staphylococci provide the growth requirements needed to facilitate the growth of the NVS.

c. A clue to the presence of NVS is a positive Gram’s stain, but negative cultures.

9. Treatment of streptococcal and enterococcal infections. Most species of Streptococcus are susceptible to penicillin. S. agalactiae is less susceptible than group A and may require a combination of ampicillin and an aminoglycoside. Group D is susceptible to penicillin, whereas Enterococcus is usually resistant. Enterococcus is often treated with a penicillin-aminoglycoside combination (synergy). Some isolates are resistant to this combination therapy. Although most pneumococcal isolates are susceptible to penicillin, some strains have shown resistance. Resistant streptococcal strains are often treated with erythromycin. Linezolid is often used for treatment of infections caused by vancomycin-resistant enterococci (VRE).

C. Streptococcus-like organisms (Table 7–3)

1. *Aerococcus* is very similar to *Enterococcus* on blood agar. The gram-positive coccus is susceptible to vancomycin and can be isolated from tissue samples of endocarditis and other varied infections.

2. *Leuconostoc* is very similar to viridans streptococci on blood agar. It is found in the general environment. A Gram’s stain shows gram-positive coccobacilli in pairs and short chains. *Leuconostoc* has been found in patients who have meningitis and endocarditis. It is intrinsically resistant to vancomycin.

3. *Pediococcus* is also found in the general environment. A Gram’s stain shows gram-positive cocci in pairs, tetrads, and clusters. *Pediococcus* is a rare isolate in patients who have septicemia. The bacterium is intrinsically resistant to vancomycin.

D. Laboratory identification of Streptococci

1. Hemolysis on blood agar is an important characteristic (Table 7–2). (See Web Color Images 7–17, 7–18, and 7–19.)

2. Bile solubility measures autolysis of bacteria under the influence of a bile salt (sodium deoxycholate). *S. pneumoniae* is bile soluble.

3. Optochin (ethylhydrocuprein hydrochloride) susceptibility is determined by a zone of inhibition (>14 mm with a 5 mcg optochin disk) after growing the organism on blood agar with a filter paper disk containing optochin. Results correlate with bile solubility; that is, optochin-susceptible isolates are bile soluble. *S. pneumoniae* is optochin susceptible. (See Web Color Image 7–21.)

4. Bacitracin (Taxo A) susceptibility is a characteristic of *S. pyogenes*. The test is performed by placing a filter paper disk containing bacitracin on an inoculated blood agar plate, and measuring the zone of inhibition following incubation. (See Web Color Image 7–23.)

5. Group A and B streptococci are resistant to sulfamethoxazole-trimethoprim (SXT). This resistance can be measured with a filter paper disk or by incorporating SXT into blood agar. The latter technique allows for selective isolation. *Enterococcus* species are also SXT-resistant.

6. Group B streptococci hydrolyze hippurate. The glycine liberated can be detected by triketohydrindene hydrate (Ninhydrin), which imparts a purple color.

7. The Christie, Atkins, and Munch-Petersen (CAMP) test presumptively identifies group B streptococci by measuring the enhanced hemolytic activity of staphylococcal β-lisolysin by *S. agalactiae*. Group B streptococci, plated perpendicular to *S. aureus*, demonstrate a characteristic arrow-shaped hemolysis pattern. (See Web Color Image 7–25.)
8. The ability of an organism to hydrolyze esculin is the basis of the esculin test. A positive result is a black precipitate in the agar surrounding the growth. Group D streptococci and Enterococcus are bile esculin positive. (See Web Color Image 7–26.)

9. Enterococcus is able to grow in nutrient broth containing 6.5% NaCl. (See Web Color Image 7–26.)

10. Hydrolysis of PYR can be detected by the development of a red color on the addition of cinnamaldehyde reagent. This test is specific for Enterococcus and S. pyogenes. (See Web Color Image 7–24.)

11. The LAP test (leucine aminopeptidase) is used to help differentiate Aerococcus and Leuconostoc from the other Streptococcus species. Both bacteria are LAP-negative, while other streptococci are LAP-positive. LAP hydrolyzes the substrate, leucine-β-naphthylamide, to β-naphthylamine. Development of a red color is detected upon addition of DMACA.

12. Serology testing for detection of the C carbohydrate of the cell wall is used for serogrouping of the β-hemolytic streptococci.

IX. AEROBIC GRAM-POSITIVE BACILLI

A. General characteristics

1. The members of this group that are seen most frequently in the clinical laboratory are listed in Box 7–1.

2. Except for Corynebacterium diphtheriae, these organisms are of low pathogenicity and usually require an immunocompromised host.

3. With the exception of Bacillus, these organisms are all pleomorphic rods, and most grow well on standard media.

B. Listeria monocytogenes is widespread in the environment. It causes a wide variety of infections, especially in neonates, pregnant women, and immunosuppressed persons. Meningitis is a common outcome.

1. Isolation is usually from blood, CSF, or swabs of lesions. L. monocytogenes grows well on blood agar and closely resembles group B streptococcus. Growth occurs at 4°C. This allows the use of the cold enrichment technique, which requires inoculation of the specimen into broth medium, followed by incubation at 4°C for several weeks. This technique has limited clinical importance.

2. Gram stain shows a gram-positive rod or coccobacillus. (See Web Color Image 7–27.)

3. The bacterium is catalase-positive, hydrolyzes hippurate and esculin, and is CAMP test-positive with block hemolysis. (See Web Color Image 7–28) A characteristic tumbling motility is demonstrated when the organism is grown in broth at room temperature and umbrella motility is observed in semisolid agar motility medium following room temperature incubation. (See Web Color Image 7–29.) Identification of this organism is summarized in Box 7–2.

C. Erysipelothrix rhusiopathiae is an uncommon isolate. It is a pleomorphic gram-positive bacillus that often forms long filaments (see Web Color Image 7–30). The usual route of infection is through the skin. It is catalase negative, and forms hydrogen sulfide. The bacterium is nonmotile, but produces a characteristic “bottle brush” extension laterally from the streak line in soft gelatin agar (see Web Color Image 7–31).

<table>
<thead>
<tr>
<th>Box 7–1</th>
<th>Most Commonly Isolated Aerobic Gram-Positive Bacilli</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacillus</td>
<td></td>
</tr>
<tr>
<td>Corynebacterium</td>
<td></td>
</tr>
<tr>
<td>Erysipelothrix</td>
<td></td>
</tr>
<tr>
<td>Listeria</td>
<td></td>
</tr>
<tr>
<td>Nocardia</td>
<td></td>
</tr>
</tbody>
</table>
CHAPTER 7 Clinical Microbiology

Box 7–2 Characteristics of Listeria monocytogenes

<table>
<thead>
<tr>
<th>Characteristic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gram-positive coccobacillus</td>
</tr>
<tr>
<td>Umbrella motility pattern (motility agar tube) at room temperature</td>
</tr>
<tr>
<td>Hippurate hydrolysis positive</td>
</tr>
<tr>
<td>CAMP test positive–block hemolysis pattern</td>
</tr>
<tr>
<td>Esculin hydrolysis positive</td>
</tr>
<tr>
<td>Growth at 4°C</td>
</tr>
<tr>
<td>Catalase positive</td>
</tr>
<tr>
<td>β hemolytic (very similar to group B streptococcus)</td>
</tr>
</tbody>
</table>

D. The most important species of Corynebacterium are C. diphtheriae, C. jeikeium, and C. urealyticum.

1. All species are pleomorphic, paliading, gram-positive bacilli and resemble C. diphtheriae on Gram’s stain. The morphology was, therefore, termed diphtheroid. The morphology may also be described as picket fence or Chinese letters (see Web Color Image 7–32).

2. C. diphtheriae is the cause of diphtheria. The disease has a presentation of local inflammation of the throat with a pseudomembrane caused by dead cells and exudate. The diphtheria toxin damages major organ systems and results in a high mortality rate when infected persons go untreated. Diphtheria occurs in nonimmunized populations. Treatment is with an antitoxin.

a. Laboratory diagnosis consists of culture and testing for toxin production. Media that have been developed for the growth and identification of C. diphtheriae are summarized in Table 7–4. Suspicious colonies from cystine-tellurite or Tinsdale’s agar are gram stained. Catalase and urease tests are performed on gram-positive rods with diphtheroid morphology. (Loeffler’s agar can be used to enhance the pleomorphic microscopic morphology of the gram-positive rods.). Urease negative isolates are presumptively identified as C. diphtheriae.

b. Toxin production may be determined by the Elek test, which detects toxin production by an isolate using an antitoxin-impregnated filter paper strip that is laid perpendicular to lines of bacterial growth. Precipitin lines are formed if the test strain of C. diphtheriae is a toxigenic strain.

3. C. jeikeium (group JK) is an extremely virulent organism. It may cause infections following implantation of prosthetic devices, and it is resistant to a wide range of antibiotics. This organism is suspected in those patients who are immunocompromised or have undergone invasive procedures or in whom an isolate with typical diphtheroid morphology is found.

4. C. urealyticum is a urinary pathogen that is slow growing (48 hours) and strongly urease positive. Urease production occurs within minutes following inoculation on a urea slant.

5. Differentiation between Corynebacterium, Erysipelothrix, and Listeria is outlined in Table 7–5.

E. The two species of Bacillus that are of medical importance are B. anthracis and B. cereus. These aerobic bacteria are catalase-positive, gram-positive spore formers that appear singly or in chains with a “boxcar” morphology on Gram’s stain (see Web Color Image 7–33).

1. B. anthracis is the cause of anthrax, a rare disease in the United States. It usually appears in the cutaneous form as a result of wounds contaminated with anthrax spores.

### Table 7–4 Media Used to Isolate Corynebacterium diphtheriae

<table>
<thead>
<tr>
<th>Media</th>
<th>Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cystine-tellurite agar</td>
<td>Colonies are black or gray</td>
</tr>
<tr>
<td>Tinsdale’s agar</td>
<td>Colonies are dark brown-to-black with brown-to-black halos</td>
</tr>
<tr>
<td>Loeffler agar</td>
<td>Supports growth and enhances pleomorphism</td>
</tr>
</tbody>
</table>
The lesion that is formed develops a characteristic center of necrosis, which has been termed a black eschar or malignant pustule. Handling of *B. anthracis* is extremely dangerous and should only be done within a biologic safety cabinet.

2. *B. cereus* is a common cause of food poisoning.
3. Laboratory identification is accomplished by growth on blood agar. Colonies of both species are large and flat. *B. cereus* is β-hemolytic. The differentiation between *B. anthracis* and *B. cereus* is outlined in Table 7–6.

### F. Aerobic Actinomycetes

include *Nocardia*, *Actinomadura*, and *Streptomyces* species. These microorganisms are similar morphologically to fungi. They are gram-positive bacilli, but do not produce spores.

1. *Nocardia* species are saprophytes and are found worldwide in soil and on plant material. They cause pulmonary and cutaneous infections in humans. *Nocardia asteroides* is the most commonly isolated member of *Nocardia*. This organism is usually found in immunocompromised patients as a chronic infection, particularly pulmonary. It is pleomorphic and partially acid-fast. (See Web Color Image 7–34). Growth is slow (up to 6 weeks). The colonial morphology is dry and heaped, similar to a fungus (see Web Color Images 7–35 and 7–36). It also has a soil or musty-basement odor. Exudates may demonstrate "sulfur granules," which are masses of filamentous organisms with pus materials.

2. *Actinomadura* are etiologic agents of mycetomas. Their microscopic and macroscopic morphology is similar to that of the *Nocardia* species. However, the microorganisms are not acid-fast.

3. *Streptomyces* species are primarily saprophytes, found as soil organisms. They may cause opportunistic infections similar to those caused by the other aerobic actinomycetes.

### G. *Lactobacillus* species

is a pleomorphic, nonspore-forming, gram-positive bacillus. It is catalase-negative, and may grow better under anaerobic conditions. *Lactobacilli* are normal vaginal flora.

### H. *Arcanobacterium haemolyticum* is a small, β-hemolytic, nonspore-forming, gram-positive bacillus. It resembles the β-hemolytic streptococci. The organism is the etiologic agent of pharyngitis and must be differentiated from *Streptococcus pyogenes*. It is catalase-negative and exhibits a reverse CAMP reaction.

### X. NEISSERIA AND MORAXELLA CATARRHALIS

#### A. General characteristics

1. Members of this group are gram-negative cocci that are often seen in pairs. The adjacent sides are flattened, producing a kidney-bean shape (see Web Color Image 7–37).
2. They are all oxidase-positive and catalase-positive (see Web Color Images 7–38 and 7–41). Differentiation of species is often based on acid production from carbohydrate utilization.

3. Some of the pathogenic species may have fastidious growth requirements.

4. Proper specimen collection is essential for successful isolation. N. gonorrhoeae and N. meningitidis are especially sensitive to drying, cold, and chemicals (disinfectants and antiseptics). Dacron or rayon swabs are less inhibitory than calcium alginate and cotton and are preferred for collection. Specimens should be kept at room temperature and plating should occur as soon as possible following collection.

B. Neisseria gonorrhoeae

1. Infections
   a. Gonorrhea is a frequently seen venereal disease. The majority of infected men show symptoms such as burning and discharge from the urethra (gonococcal urethritis). Females may be asymptomatic, but infection in this gender may lead to pelvic inflammatory disease. Gonorrhea may cause sterility in males and females and disseminate to blood, skin, and joints.
   b. Ophthalmia neonatorum is primarily a gonococcal infection of the conjunctiva of newborns as a result of passage through an infected mother’s birth canal. It can be successfully treated at birth to prevent blindness.
   c. Penicillinase-producing Neisseria gonorrhoeae are a common problem. A β-lactamase test should be done on each isolate to detect resistance to the penicillin antibiotics.

2. Direct microscopic examination of gram stained urethral discharge from males is a valuable diagnostic procedure. The presence of gram-negative intracellular diplococci (see Web Color Image 7–37) from a symptomatic male with discharge has a 95% correlation rate with culture and is strong presumptive evidence of gonorrhea. Correlation is much lower in females because of the normal flora.

3. Culture of N. gonorrhoeae can be accomplished with various enriched and selective media.
   a. Specimens for culture should be collected from infected sources (cervix, urethra, rectum, or throat.) Swabs or exudates are acceptable specimens, and should be kept at room temperature prior to processing.
   b. Agar. N. gonorrhoeae grows on chocolate agar, but not on blood agar. In those cases in which normal flora may contaminate the medium, selective agars, inhibitory to organisms other than Neisseria species, may be used.
      (1) Selective media include Thayer-Martin and modified Thayer-Martin (MTM), Martin-Lewis, and New York City.
      (2) These media are often packaged in self-contained transport/incubation systems that are inoculated at the site of collection. Examples include Transgrow, Gono-Pak, and JEMBEC plates.
   c. Culture and identification. Plates are incubated at 35°C in a 3% to 5% carbon dioxide, humidified atmosphere. Colonies of N. gonorrhoeae are flat, smooth, glossy, and gray to white. The oxidase-positive, catalase-positive, gram-negative diplococcus produces acid from glucose utilization. Identification/differentiation is outlined in Table 7–7. Various tests are available for direct detection of

| Table 7–7 | Differentiation of Neisseria and Moraxella catarrhalis |
| Organisms | Oxidase | Catalase | Acid from Glucose | Acid from Maltose | DNAse | Growth on Nutrient Agar | Butyrate Esterase |
| N. gonorrhoeae | + | + | + | − | − | − | − |
| N. meningitidis | + | + | + | + | − | − | − |
| Other Neisseria species | + | + | + | + | + | + | + |
| M. catarrhalis | + | + | − | − | + | + | + |

DNAse = deoxyribonuclease; V = variable; + = positive; − = negative.
A Concise Review of Clinical Laboratory Science

*N. gonorrhoeae* from clinical specimens. These include detection of cellular antigens and those that detect gonococcal nucleic acid.

**C. Neisseria meningitidis**

1. **Infections**
   a. Meningococcal meningitis is transmitted by respiratory droplets. Prolonged close contact is necessary for infection. The onset is abrupt, with headache, stiff neck, and fever. Petechial skin lesions may be present.
   b. Meningococccemia, inflammation of both the brain substance and surrounding membranes, also involves the blood vessels and various major organs. Petechial skin lesions are common. This condition may progress to DIC.

2. **Direct microscopic examination** of CSF sediment may reveal intracellular and extracellular gram-negative diplococci (see Web Color Image 7–37).

3. **Specimens** for culture include CSF, blood, and joint fluid. Nasopharyngeal swabs are cultured to detect carriers. Specimens should be kept at room temperature and processed as soon as possible. Swabs should not be allowed to dry out. Growth is seen on blood and chocolate agar and is enhanced with increased carbon dioxide and humidity. Colonies of *N. meningitidis* are flat, smooth, glistening, and gray to white. Acid production from the utilization of glucose and maltose differentiates the bacterium from *N. gonorrhoeae*.

Identification is outlined in Table 7–7.

**D. Moraxella catarrhalis** is part of the normal upper respiratory tract flora. It may cause otitis media, sinusitis, and respiratory infections. *M. catarrhalis* grows on blood, chocolate, and nutrient agars. Identification of the asaccharolytic gram-negative coccus is outlined in Table 7–7.

**E. Other Neisseria species** only rarely cause clinical infection. These species may need to be differentiated from the pathogenic species, because they are normal flora at the same sites at which the pathogenic species are located (Table 7–7).

**XI. MISCELLANEOUS GRAM-NEGATIVE BACILLI**

**A. Haemophilus**

1. **Members of the genus Haemophilus** are gram-negative, nonmotile bacilli and cocacobacilli, which are often pleomorphic. Members of *Haemophilus* require hemin (X factor) and/or nicotinamide adenine dinucleotide (NAD; V factor) for growth (except *H. aphrophilus*). These fastidious bacteria grow on chocolate agar, but not on blood agar, due to its absence of the NAD.

2. **H. influenzae** is the most important species, causing meningitis, otitis media, epiglottitis, pneumonia, and contagious conjunctivitis. Children, especially those who have not been immunized, are particularly at risk. *H. influenzae* is part of the normal upper respiratory tract flora. Capsular serotype b is the most common cause of disseminated infections, but widespread immunization with the Hib vaccine has resulted in a decreased incidence of these infections.

3. **H. ducreyi** is the cause of chancroid, a sexually transmitted disease. Organisms enter through breaks in the skin and multiply locally. Approximately 1 week later, a small papule appears that soon develops into a painful ulcer. A Gram’s stain of the lesion exudate may show small pleomorphic gram-negative bacilli in clusters (“school of fish” morphology).

4. **H. aegyptius** (Koch-Weeks bacillus) is associated with acute, contagious conjunctivitis, commonly referred to as “pinkeye.”

5. **H. parainfluenzae** and *H. aphrophilus* are human normal oral flora and are seen primarily in endocarditis.

6. **Differentiation** of the *Haemophilus* species is summarized in Table 7–8.

a. X and V factor requirements can be determined by placing X and V factor–impregnated filter paper strips onto a nutrient agar plate that has been inoculated
Table 7–8 Differentiation of Haemophilus Species

<table>
<thead>
<tr>
<th>Species</th>
<th>Growth-Factor Requirement</th>
<th>Porphyrin</th>
<th>Catalase</th>
<th>Fermentation of:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>X</td>
<td>V</td>
<td></td>
<td>Glucose</td>
</tr>
<tr>
<td>H. influenzae</td>
<td>+</td>
<td>+</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>H. parainfluenzae</td>
<td>−</td>
<td>+</td>
<td></td>
<td>−</td>
</tr>
<tr>
<td>H. ducreyi</td>
<td>+</td>
<td>−</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>H. aphrophilus</td>
<td>−</td>
<td>−</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>H. aegyptius</td>
<td>+</td>
<td>+</td>
<td></td>
<td>−</td>
</tr>
</tbody>
</table>

V = variable; + = positive; − = negative.

with the unknown species. X and V requirements are determined by growth patterns (e.g., growth around X indicates a requirement for that factor). As depicted in Web Color Image 7–39, H. influenzae requires both X and V factors for growth. H. parainfluenzae requires only V factor for growth (see Web Color Image 7–40).

b. Growth can also be seen surrounding colonies of S. aureus, S. pneumoniae, or Neisseria species. These organisms produce V factor as a metabolic byproduct and lyse the RBCs in the medium, releasing the X factor. This is called the satellite phenomenon (see Web Color Image 7–41).

c. The porphyrin test is a sensitive method to determine X factor requirements. Those species that do not require X factor yield a positive porphyrin test (e.g., H. influenzae is porphyrin negative).

d. Fermentation of carbohydrates (glucose, sucrose, lactose) can aid in identification of the species.

e. H. influenzae may be divided into biotypes. The site of infection by H. influenzae can be correlated with biotype. Biotypes are determined by an isolate’s activity with indole, urease, and ornithine decarboxylase. The disease association and biochemical characteristics of common biotypes are summarized in Table 7–9. Biotype I is most commonly associated with meningitis and epiglottis. Because of similar biochemical characteristics, additional testing is necessary to differentiate H. aegyptius and H. influenzae.

7. Antibiotic resistance. Resistance to the penicillins is common due to β-lactamases and other mechanisms.

B. Pasteurella multocida is the most commonly encountered species in the Pasteurella genus. It is found as normal flora in the respiratory tract of animals, especially dogs and cats. Isolation of P. multocida is always a strong possibility in an infected dog or cat bite or scratch. This species appears as a small gram-negative coccobacillus with bipolar staining. The key characteristics for differentiating P. multocida are listed in Box 7–3.

C. Bordetella species are very small gram-negative bacilli. There are three species that cause human infection: B. pertussis, B. parapertussis, and B. bronchiseptica. All cause respiratory tract infection, but B. pertussis causes the most serious infection, which is whooping cough.

1. Bordetella pertussis, the cause of pertussis or whooping cough, is found worldwide and is spread via droplets.

   a. Disease progression. The disease begins with coldlike symptoms (i.e., runny nose, sneezing, malaise). This catarrhal stage is the most infectious. After 1 to 2 weeks,
Box 7–3 Key Characteristics of Pasteurella multocida

- Gram-negative coccobacillus with bipolar staining
- Growth on blood agar (may have a musty or mousey smell)
- No growth on MacConkey agar
- Catalase positive
- Indole positive
- Oxidase positive
- Penicillin susceptible (2–U disk)
- Glucose utilization

the paroxysmal stage begins, with violent coughs that often make it difficult for the infected person to take a breath. Convalescence can take weeks to months with secondary complications, such as pneumonia, seizures, and encephalopathy possible.

b. Specimen collection and transport. B. pertussis is very sensitive to drying and to trace toxic chemicals on swabs and in media. Specimen collection and transport must be done correctly for successful culture. Cotton swabs are toxic; calcium alginate or Dacron swabs should be used. The best specimen is a nasopharyngeal swab or aspirate. Immediate plating is preferred, because the organism does not readily survive transport. A swab should also be collected for a direct fluorescent antibody and Gram’s stain of smears.

1. Growth can be accomplished using Regan-Lowe or Bordet-Gengou agar (charcoal-horse blood agar). B. pertussis colonies, often described as “mercury drop” colonies, are small and pearl-like in appearance after 3 to 4 days. It does not grow on sheep blood agar and is urease-negative.

2. Identification is by microscopic and colonial morphology on selective media, biochemical reactions, and reactivity with specific antiserum, usually in a direct fluorescence test (DFA) (see Web Color Image 7–42).

2. B. parapertussis can be found in patients who have respiratory tract illness that resembles a mild form of pertussis. This species grows on sheep blood agar within 2 to 3 days and is urease positive within 24 hours. B. bronchiseptica is rarely found in humans but causes respiratory tract disease in animals (“kennel cough”). Growth is observed on sheep blood agar within 1 to 2 days and it is urease-positive within 4 hours (see Web Color Image 7–43).

D. Francisella tularensis is the causative agent of tularemia, a zoonotic disease. Transmission is via contact with infected animals (e.g., rabbits, deer), arthropod bites (e.g., ticks, fleas), or inhalation. Isolation generally requires extended incubation on media enriched with cystine or cysteine. The gram-negative rod/coccobacillus is very small and stains poorly on Gram’s stain. Definitive identification is made with specific antisera (direct fluorescence). This organism is very dangerous to work with in the laboratory. It should always be handled under a biologic safety hood with safety precautions strictly observed.

E. Members of the genus Brucella cause disease in animals. Human disease is normally a result of contact with the animals or their waste, meat, hides, or secretions. There are four species responsible for the majority of human disease: B. melitensis, B. abortus, B. suis, and B. canis.

1. The disease caused is brucellosis (also known as Bang’s disease or undulant fever). It is characterized by fever, chills, fatigue, weakness, and internal organ lesions. It can be chronic. Brucellosis is a CDC reportable infection.

2. Brucella species are most often isolated from blood or bone marrow. The organism is slow-growing, and blood cultures may need to be incubated for 4 to 6 weeks before they are considered negative. A Gram’s stain shows a faintly staining, small, gram-negative coccobacillus. Serologic tests (agglutination of the isolate) are valuable in identification. The CO₂ requirement, along with urease, hydrogen sulfide, and
Table 7–10 Differentiation of Brucella Species

<table>
<thead>
<tr>
<th>Species</th>
<th>CO₂ Required</th>
<th>Urease</th>
<th>H₂S</th>
<th>Thionine</th>
<th>Fuchsin</th>
<th>Natural Hosts</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. abortus</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>Cattle</td>
</tr>
<tr>
<td>B. melitensis</td>
<td>−</td>
<td>+</td>
<td>V</td>
<td>−</td>
<td>−</td>
<td>Goats, sheep</td>
</tr>
<tr>
<td>B. suis</td>
<td>−</td>
<td>+</td>
<td>&lt;0.5h</td>
<td>+</td>
<td>−</td>
<td>Swine</td>
</tr>
<tr>
<td>B. canis</td>
<td>−</td>
<td>+</td>
<td>&lt;0.5h</td>
<td>−</td>
<td>−</td>
<td>Dogs</td>
</tr>
</tbody>
</table>

H₂S = hydrogen sulfide; + = positive; – = negative; V = variable

growth in the presence of thionin and basic fuchsin can also be used in differentiation of the species. Table 7–10 summarizes the differential characteristics of the Brucella species. Brucella organisms should be handled under biosafety level 3 conditions with safety precautions strictly observed.

F. *Antinobacillus actinomycetemcomitans* is a slow-growing, small, facultative gram-negative bacillus associated with endocarditis, bacteremia, and dental infections. It may be associated with *Actinomyces*. It is catalase positive and oxidase negative (Table 7–11).

G. *Kingella kingae* colonizes the upper respiratory tract and is primarily associated with infections of bones and joints, as well as endocarditis in children and young adults. It grows on sheep blood agar and is β-hemolytic. A Gram’s stain shows short, plump, gram-negative rods with square ends (Table 7–11). Other less pathogenic species in the genus are *K. denitrificans* and *K. oralis*.

H. *Capnocytophaga* is normal oral flora in humans. It may cause serious infections in immunosuppressed patients. It has been associated with dog bites and is usually isolated from blood or cerebrospinal fluid. It grows on sheep blood agar, but is capnophilic and must have CO₂ for growth. Colonies are beige or yellow and show a haze of growth at the periphery as a result of gliding motility (see Web Color Image 7–44). Gram’s stain shows fusiform, gram-negative bacillus (Table 7–11). (See Web Color Image 7–45.)

I. *Cardiobacterium hominis* is a pleomorphic, gram-negative rod that grows on blood, but not MacConkey agar. It is normal human oral flora and is found in patients who have endocarditis and bacteremia. The colonies are small, and growth is slow. Differentiation of the miscellaneous gram-negative bacilli and coccobacilli is summarized in Table 7–11.

Table 7–11 Differentiation of Gram-Negative Bacilli/Coccobacilli

<table>
<thead>
<tr>
<th>Organism</th>
<th>Oxidase</th>
<th>Catalase</th>
<th>Coccolid</th>
<th>Fusiform</th>
<th>Indole</th>
<th>Urease</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Antinobacillus actinomycetemcomitans</em></td>
<td>−</td>
<td>V</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td><em>Capnocytophaga</em></td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td><em>Cardiobacterium hominis</em></td>
<td>+</td>
<td>−</td>
<td>Pleomorphic</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td><em>Kingella kingae</em></td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td><em>Pasteurella multocida</em></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>Brucella</em></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>+ 2h</td>
</tr>
<tr>
<td><em>Franciscella tularensis</em></td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

= positive; − = negative
XII. ENTEROBACTERIACEAE

A. General characteristics
1. The Enterobacteriaceae is the largest and most medically important family of gram-negative bacilli (see Web Color Image 7–4). Although there are numerous genera and species, more than 90% of the medically important isolates belong to just a few genera (Box 7–4).
2. The Enterobacteriaceae are found worldwide and are part of the normal flora of all animals. They are a common cause of nosocomial infections.
3. Commonly seen sites of infection with members of the Enterobacteriaceae are listed in Table 7–12.
4. All members ferment glucose, reduce nitrates to nitrites, and are oxidase-negative. The oxidase reaction is especially important in making a rapid distinction between the Enterobacteriaceae and the majority of the gram-negative nonfermenters. However, the oxidase-positive Plesiomonas shigelloides, formerly in the family Vibrionaceae, has been moved to the family Enterobacteriaceae. Phylogenetic studies have shown that Plesiomonas is closely related to members in this family, particularly the genus Proteus. (Identification of P. shigelloides will be discussed in Section XIII.)
5. Antigens of this group include the O or somatic antigen, found in the bacterial cell wall, the H or flagellar antigen, and the K or capsular antigen.

B. Various culture media and tests are used for the isolation, selection, differentiation, and identification of the Enterobacteriaceae.
1. Media used for isolation and selection of Salmonella and Shigella, along with expected reactions, are summarized in Table 7–13. (see Web Color Images 7–46, 7–47, and 7–48).
2. Media used for the isolation and detection of lactose fermenters are summarized in Table 7–14. (see Web Color Images 7–49, 7–50, and 7–51).

<table>
<thead>
<tr>
<th>Table 7–12 Sites of Infections with Common Enterobacteriaceae</th>
</tr>
</thead>
<tbody>
<tr>
<td>Site</td>
</tr>
<tr>
<td>Blood</td>
</tr>
<tr>
<td>Central nervous system</td>
</tr>
<tr>
<td>Gastrointestinal</td>
</tr>
<tr>
<td>Lower respiratory tract</td>
</tr>
<tr>
<td>Urinary tract</td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>

Box 7–4 Medically Significant Enterobacteriaceae

- Escherichia
- Shigella
- Salmonella
- Klebsiella
- Enterobacter
- Serratia
- Proteus
**Table 7–13 Media Used to Isolate and Select Salmonella and Shigella**

<table>
<thead>
<tr>
<th>Media</th>
<th>Characteristic Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bismuth sulfite agar</td>
<td>Salmonella typhi are black colonies surrounded by a black zone of precipitate</td>
</tr>
<tr>
<td>Brilliant green agar</td>
<td>Proteus colonies are red to pink, Salmonella colonies are red to pink, Shigella is inhibited</td>
</tr>
<tr>
<td>Gram-negative broth</td>
<td>The growth of most gram-negative rods is inhibited, whereas Salmonella and Shigella growth is enriched</td>
</tr>
<tr>
<td>Hektoen enteric agar</td>
<td><em>Escherichia coli</em> and lactose/sucrose fermenters are orange to salmon pink colonies</td>
</tr>
<tr>
<td></td>
<td>Proteus is usually inhibited, Salmonella colonies are blue to blue-green, with black centers if hydrogen sulfide is produced</td>
</tr>
<tr>
<td></td>
<td>Shigella colonies are green</td>
</tr>
<tr>
<td>Salmonella-Shigella agar</td>
<td><em>E. coli</em> colonies are red</td>
</tr>
<tr>
<td></td>
<td>Proteus colonies are colorless, with black centers</td>
</tr>
<tr>
<td></td>
<td>Salmonella colonies are colorless, with or without black centers</td>
</tr>
<tr>
<td></td>
<td>Shigella colonies are colorless</td>
</tr>
<tr>
<td>Selenite broth</td>
<td><em>E. coli</em> stool specimens are enriched</td>
</tr>
<tr>
<td></td>
<td>Gram-positive organisms are inhibited</td>
</tr>
<tr>
<td>Xylose-lysine-deoxycholate agar</td>
<td><em>E. coli</em> colonies are yellow</td>
</tr>
<tr>
<td></td>
<td>Proteus colonies are clear or yellow</td>
</tr>
<tr>
<td></td>
<td>Salmonella colonies are red, with black centers</td>
</tr>
<tr>
<td></td>
<td>Shigella colonies are clear</td>
</tr>
</tbody>
</table>

3. Media and tests used for the identification of the Enterobacteriaceae include the following:

a. **Triple sugar iron agar (TSI).** This medium differentiates gram-negative bacilli by their ability to ferment glucose, lactose, and sucrose and to produce hydrogen sulfide (Web Color Image 7–52).
   (1) **Nonfermenters** produce an alkaline slant and alkaline deep (no change in the red color of the medium).
   (2) **Nonlactose fermenters** produce an alkaline slant (red) and acid (yellow) deep.
   (3) **Lactose fermenters and sucrose fermenters** produce an acid (yellow) slant and acid (yellow) deep. A lactose-negative, sucrose-positive organism will produce an acid slant and acid deep.
   (4) **Hydrogen sulfide (H₂S) production** is indicated by a black precipitate in the medium.
   (5) **Kligar’s iron agar (KIA)** is a similar medium, but only incorporates the carbohydrates glucose and lactose. H₂S production can also be detected with this formulation (see Web Color Image 7–52).

b. **Nitrate reduction.** Nitrate test medium is inoculated and incubated overnight to determine the ability of microorganisms to reduce nitrates to nitrite. The presence of nitrates in the medium is detected by the addition of N,N-Dimethyl-α-naphthylamine and sulfanilic acid. The formation of a red color after the addition of the two reagents indicates that nitrite is present (positive test). If a red color is

**Table 7–14 Media Used for the Isolation and Detection of Lactose Fermenters**

<table>
<thead>
<tr>
<th>Media</th>
<th>Characteristic Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eosin-methylene blue agar</td>
<td><em>E. coli</em> colonies are dark, with a green metallic sheen, Gram-positive organisms are inhibited</td>
</tr>
<tr>
<td></td>
<td>Lactose fermenters show pink to red colonies</td>
</tr>
<tr>
<td></td>
<td>Lactose nonfermenters have colorless colonies</td>
</tr>
<tr>
<td>MacConkey agar</td>
<td>Gram-positive organisms are inhibited</td>
</tr>
<tr>
<td></td>
<td>Lactose-fermenter colonies are pink to red</td>
</tr>
<tr>
<td></td>
<td>Lactose-nonfermenter colonies are colorless</td>
</tr>
</tbody>
</table>
not detected, all available nitrate may have been reduced to nitrite and then completely converted to nitrogen gas (N₂), nitric oxide (NO), or nitrous oxide (N₂O). No nitrite remains to react with the sulfanilic acid. This can be detected by adding a small pinch of zinc dust to the tube (metallic zinc reduces nitrate to nitrite). With the formation of nitrite upon addition of zinc, the reactions can take place, resulting in the red color. A red color at this point indicates that nitrate was still in the broth (negative test). Absence of red color after the addition of zinc indicates that no nitrate was left (positive test). All Enterobacteriaceae reduce nitrites to nitrites (see Web Color Image 7–53).

c. Oxidase. The oxidase test determines the presence of the cytochrome oxidase system that oxidizes reduced cytochrome with molecular oxygen. A positive test is the development of a purple when the bacteria is mixed with the reagent tetramethyl-p-phenylenediamine dihydrochloride. The Enterobacteriaceae are oxidase-negative, with the exception of Plesiomonas species (see Web Color Image 7–38).

d. Indole. The indole test detects an organism’s ability to produce the enzyme tryptophanase and deaminate tryptophan to indole, pyruvic acid, and ammonia. A positive reaction is a detected with the addition of Ehrlich’s reagent, paradimethyl-laminobenzaldehyde, or Kovac’s reagent. A positive reaction is a pink color (see Web Color Images 7–54 and 7–55).

e. Methyl red-Voges-Proskauer (MR-VP) broth. The MR and VP tests are used to determine the method by which bacteria metabolize glucose: the mixed acid fermentation pathway or the butylene glycol pathway. The tests detect the end products of glucose fermentation, in accordance with the pathway an organism uses to metabolize glucose. Isolates are inoculated into MR-VP broth and allowed to grow for 48 hours. At that time, the broth is split into two fractions: one to measure methyl red and one for the Voges-Proskauer test (see Web Color Images 7–54 and 7–55).

(1) Those organisms that carry out mixed acid fermentation produce vast amounts of acid that will convert the methyl red indicator to a red color (pH <4.4).

(2) The Voges-Proskauer test measures the production of acetoin. The addition of 40% potassium hydroxide followed by α-naphthol results in a red complex (neutral pH), which indicates a positive test.

f. Citrate. The citrate test determines whether an organism can utilize sodium citrate as a sole source of carbon. Ammonium salts are the nitrogen source in the medium and utilization of these salts results in the release of ammonia, causing a pH change. The bromothymol blue indicator turns the medium from green to blue (a positive test). (See Web Color Images 7–54 and 7–55.)

g. Urea. Organisms that produce urease will hydrolyze urea to form ammonia. The ammonia reacts in solution to form ammonium carbonate, which increases the pH. This is detected by the phenol red in the medium and turns the medium a bright pink color (positive test). Christensen’s urea agar is generally a preferred test medium. (See Web Color Image 7–43.)

h. Gelatin hydrolysis. Bacteria that produce gelatinases that break down gelatin into amino acids. A slant of nutrient gelatin is inoculated and incubated at room temperature for up to several days. If incubation is at 35°C, the medium should be chilled in a refrigerator before reading the test results. Liquefaction of the gelatin is a positive test.

i. Hydrogen sulfide (H₂S). A bacterium utilizes the sodium thiosulfate sulfur source to form H₂S, a colorless gas. H₂S combines with the indicator, ferrous sulfate. Numerous media demonstrate the production of H₂S (e.g., sulfide-indole-motiligy agar, motility-indole-ornithine agar, Hektoen enteric agar, Salmomella-Shigella agar, Triple sugar iron agar, Kligler Iron agar, and lysine iron agar). (See Web Color Images 7–46, 7–47 and 7–56.)

j. Phenylalanine deaminase. The deamination of phenylalanine (removal of an amine group) results in the production of phenylpyruvic acid. Following overnight incubation and the addition of a 10% ferric chloride to an inoculated slant, a green
color indicates the presence of phenylpyruvic acid, a positive test. *Proteus*, *Mor-
ganella*, and *Providencia* species are phenylalanine deaminase-positive. (See Web Color Image 7–57.)

k. Decarboxylase and dihydrolyase tests. The decarboxylation (removal of a car-
bboxyl group, COOH) of *lysine*, *ornithine*, and *arginine* (dihydrolyase reaction) may be detected by inoculating media with a specific amino acid and glucose, a carbohydrate source. Semisolid agar tubes are inoculated by stabbing. An acid pH and anaerobic environment are required for decarboxylation reactions to oc-
cur. A yellow color initially indicates glucose fermentation and results in an acid pH that is lowered enough to activate the decarboxylase enzymes. A positive test, caused by an alkaline pH shift, is a return to the original color of the uninoculated medium (purple). Text media include *Moeller decarboxylase base medium* (see Web Color Image 7–58), *motility-indole-ornithine (MIO)*, or *lysine iron agar (LIA).*

l. β-galactosidase and the orthonitrophenyl-B-D-galactopyranoside (ONPG) test. Organisms that are late or slow lactose fermenters may appear as non-
fermenters on primary media. The ONPG test determines if the organism is a slow or late lactose fermenter (e.g., lacks the permease that allows lactose to enter the cell, but has B-galactosidase, which splits lactose). Lactose nonfermenters lack the B-galactosidase. β-galactosidase acts on ONPG to form a yellow compound (positive test), which indicates that the organism is a lactose fermenter. (See Web Color Image 7–59.)

m. DNase. Bacterial DNases are endonucleases that cleave phosphodiester bonds in DNA, resulting in smaller subunits of the polynucleotide. A bacterial isolate is streaked onto a medium with 0.2% DNA and incubated for 24 hours at 35°C. Following incubation, 1N HCl is added to the surface of the medium. Unhydrolyzed DNA is insoluble in HCl and forms a precipitate. A positive reaction is the formation of a clear zone (halo) around the inoculum.

n. Motility. Motility can be demonstrated by microscopic examination of wet mounts of bacteria. In addition, semisolid medium, with agar concentrations of 0.4% or less, can be used to demonstrate motility. Following overnight incubation, move-
m ent away from the stab line or a hazy appearance throughout the medium is indicative of a positive reaction by a motile bacterium. (See Web Color Image 7–60.)

o. Carbohydrate fermentation. All Enterobacteriaceae ferment glucose, lac-
tose fermentation is used to differentiate groups of genera within the family. Other carbohydrates can be utilized by bacteria. Phenol red carbohydrates or CTA sugars with phenol red indicators are most commonly used to detect fermentation of spe-
cific carbohydrates. A yellow color indicate a fermentation and a positive reaction. (See Web Color Image 7–61.)

4. The identification of frequently encountered species in the family Enterobacteriaceae can be accomplished using the information presented in Table 7–15.

XIII. CAMPYLOBACTER, HELICOBACTER, AND VIBRIONACEAE

A. Campylobacter

1. The most clinically relevant species are *C. jejuni* subsp. *jejuni*, *C. coli*, and *C. fetus* subsp. *fetus.*

2. Diarrhea is the primary disease caused by *Campylobacter jejuni*. It is often trans-
mitted by means of contaminated water and animals, especially poultry and carcasses. *Campylobacter fetus* is the causative agent of bacteremia.

3. The characteristic gram-stain reaction and microscopic morphology of *Campylobac-
ter* is often described as faintly staining, “seagull-shaped” gram-negative rods (see Web Color Image 7–62). The organisms exhibit darting motility. Characteristics of *Campylobacter* species are summarized in Box 7–5.
### Table 7–15 Identification of the Enterobacteriaceae

<table>
<thead>
<tr>
<th>Species</th>
<th>Indole</th>
<th>Methyl Red</th>
<th>V-P</th>
<th>Citrate</th>
<th>H₂S</th>
<th>Urea</th>
<th>PDA</th>
<th>LDC</th>
<th>ODC</th>
<th>Motility (22°C)</th>
<th>Lactose (22°C)</th>
<th>Lactose (25°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em></td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td><em>Shigella</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td><em>Shigella</em></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td><em>Salmonella</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. typhi</em></td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td><em>S. paratyphi A</em></td>
<td>+</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
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</tr>
<tr>
<td><em>Citrobacter freundii</em></td>
<td>−</td>
<td></td>
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<td></td>
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<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td><em>C. diversus</em></td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td><em>Edwardsiella tarda</em></td>
<td>+</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
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</tr>
<tr>
<td><em>Klebsiella pneumoniae</em></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td><em>K. oxytoca</em></td>
<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td><em>Enterobacter aerogenes</em></td>
<td>−</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td><em>Hafnia alvei</em></td>
<td>−</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td><em>Serratia marcescens</em></td>
<td>−</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td><em>Proteus mirabilis</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td><em>P. vulgaris</em></td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Providencia rettgeri</em></td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Moraxellamorganii</em></td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Versinia enterococca</em></td>
<td>V</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Y. pestis</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Y. pseudotuberculosis</td>
<td>−</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

DNAse = deoxyribonuclease; H₂S = hydrogen sulfide; LDC = lysine decarboxylase; ODC = ornithine decarboxylase; PDA = phenylalanine deaminase; V = 10% to 89% are positive; V-P = Voges-Proskauer test; + = ≥ 90% are positive; − = ≤ 10% are positive.

4. The isolation of *Campylobacter* is accomplished by inoculating the specimen (usually stool) to *Campylobacter* blood agar. This contains several antibiotics that suppress growth of normal fecal flora. The plate is incubated in a microaerophilic atmosphere.

**Incubation at 42°C** also inhibits growth of normal fecal flora. The specimen of choice for isolation of *C. jejus* is blood with incubation at 35°C to 37°C.

5. The laboratory identification of *Campylobacter* is summarized in Figure 7–5 and Table 7–16. The oxidase-positive, catalase-positive *Campylobacter* species can be

### Box 7–5 Characteristics of Campylobacter

- Gram negative
- Curved rods—“seagull-wing-shaped”
- Darting motility
- Microaerophilic
- Oxidase positive
- Catalase positive
differentiated based on growth at 42°C, hippurate hydrolysis, and susceptibility to nalidixic acid and cephalothin. *C. jejuni* grows at 42°C, is hippurate hydrolysis-positive, susceptible to nalidixic acid, and resistant to cephalothin. Reactions for *C. fetus* are the opposite.

**B. Helicobacter** is an organism very similar to *Campylobacter*.

1. Diseases caused by *Helicobacter* include gastritis and duodenal ulcers.
2. Specimens are usually gastric biopsy material. Isolates are strongly urease-positive (see Web Color Image 7–43).

**C. The Vibrionaceae** include *Vibrio* and *Aeromonas*. *Plesiomonas*, previously a genus in the family Vibrionaceae, has been moved to the family Enterobacteriaceae. (*Plesiomonas* will be discussed in this section.)

1. The most commonly isolated species of *Vibrio* include *V. cholerae* (O-1 and non-O-1), *V. parahaemolyticus*, *V. alginolyticus*, and *V. vulnificus*.
2. All of these species are found in water sources and are transmitted by contaminated food and water.
3. *Vibrio cholerae* is the cause of *cholera*, a disease in which vast quantities of fluid and electrolytes are lost from the intestinal tract. The liquid stools are often referred to as “rice-water” stools, because they are colorless and contain mucus flecks. Cholera-causing isolates have a somatic antigen referred to as O-1. Non-O-1 isolates do not cause cholera, but may cause other infections. *V. cholerae* can be differentiated from other species by a positive “string test” when mixed with sodium deoxycholate (see Web Color Image 7–63).
4. *Vibrio parahaemolyticus* and *Vibrio alginolyticus* usually cause gastroenteritis following ingestion of raw or improperly handled seafood and wound infections following exposure to sea water. Both organisms require salt for growth (halophilic).
5. *Vibrio vulnificus* is an extremely virulent organism that causes rapidly progressive wound infections after exposure to contaminated water and septicemia after eating raw oysters.
6. The laboratory isolation and identification of *Vibrio* is summarized in Tables 7–17 and 7–18. *Vibrio* species are usually described as “curved” gram-negative rods (see Web Color Image 7–64), but this morphology is often only seen in the initial Gram stain.

**Table 7–16** Characteristics of *Campylobacter* Species

<table>
<thead>
<tr>
<th>Species</th>
<th>Growth at: 25°C</th>
<th>Hippurate Hydrolysis</th>
<th>Nalidixic Acid</th>
<th>Cephalothin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>37°C 42°C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>C. jejuni</em></td>
<td>–     +       +      +</td>
<td>S</td>
<td>R</td>
<td></td>
</tr>
<tr>
<td><em>C. coli</em></td>
<td>–     +       –      –</td>
<td>S</td>
<td>R</td>
<td></td>
</tr>
<tr>
<td><em>C. fetus</em></td>
<td>+     +       –      –</td>
<td>R</td>
<td>S</td>
<td></td>
</tr>
</tbody>
</table>

S = susceptible; R = resistant; + = positive; – = negative.
Table 7–17 Isolation of Vibrio Species on TCBS Agar

<table>
<thead>
<tr>
<th>Species</th>
<th>Colony</th>
</tr>
</thead>
<tbody>
<tr>
<td>V. cholerae O1</td>
<td>Yellow</td>
</tr>
<tr>
<td>V. cholerae non-O1</td>
<td>Yellow</td>
</tr>
<tr>
<td>V. alginolyticus</td>
<td>Yellow</td>
</tr>
<tr>
<td>V. parahaemolyticus</td>
<td>Dark blue-green</td>
</tr>
<tr>
<td>V. vulnificus</td>
<td>Dark blue-green</td>
</tr>
</tbody>
</table>

TCBS = thiosulfate-citrate-bile salts-sucrose.

of the clinical specimen. Isolated colonies are straight to pleomorphic gram-negative rods. The organisms are usually isolated from stool specimens. V. cholerae can be enriched by using alkaline peptone water (pH 8.4). This suppresses the growth of other organisms. All Vibrio species grow well on routine media. The Vibrio species are “halophilic” or “salt-loving,” and with the exception of V. cholerae and V. mimicus, all species require salt for growth. The differential medium of choice is Thiosulfate citrate bile salts sucrose (TCBS) agar (see Web Color Images 7–65 and 7–66). All isolates are indole and oxidase-positive (see Web Color Image 7–38), and have a fermentative metabolism. The reactions of the various Vibrio species on TCBS are summarized in Table 7–17.

7. *Aeromonas* and *Plesiomonas* are found in fresh and salt waters. They may cause diarrheal disease as well as other miscellaneous infections. Normally, only patients who have underlying disease are treated. This type of diarrhea diarrheal infection is usually self-limiting. The role of *Aeromonas* and *Plesiomonas* in diarrheal disease is not well established. These organisms grow well on blood and MacConkey agar, are oxidase positive, and ferment glucose. These bacteria are gram-negative rods. *Plesiomonas* may show long filamentous forms. Differentiation of *Aeromonas* and *Plesiomonas* is summarized in Table 7–19.

8. Differentiation of *Aeromonas, Plesiomonas, and Vibrio* is summarized in Table 7–20.

### XIV. GRAM-NEGATIVE NONFERMENTATIVE BACILLI

A. This large group of organisms uses biochemical pathways other than fermentation. They may be oxidizers (see Web Color Image 7–67) or they may be asaccharolytic (nonoxidizers). Gram-negative nonfermentative bacilli account for approximately 15% of gram-negative rod-shaped bacteria isolated in the clinical laboratory. They are found in the environment and cause disease in immunocompromised individuals. Virtually all of these organisms are opportunists. An isolate that grows on blood agar but not MacConkey agar should be suspected of being a nonfermenter. This is especially true if the isolate is also oxidase positive. Nonfermenters that do grow on MacConkey agar appear as lactose negative.

B. The most commonly isolated gram-negative, nonfermentative, rod-shaped bacteria are listed in Box 7–6.

C. The most frequently isolated gram-negative nonfermenter is *Pseudomonas aeruginosa*. This organism is commonly seen in patients who have serious burns and cystic fibrosis.

Table 7–18 Identification of Vibrio Species

<table>
<thead>
<tr>
<th>Species</th>
<th>Growth in NaCl</th>
<th>Acid from:</th>
<th>Susceptible to O1/129</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0%</td>
<td>8%</td>
<td>VP</td>
</tr>
<tr>
<td>V. alginolyticus</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>V. cholerae</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>V. parahaemolyticus</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>V. vulnificus</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
</tbody>
</table>

V = variable; VP = Voges-Proskauer; O1/129 = 2,4-diamino-6,7-diisopropylpteridine phosphate; + = positive; – = negative.
CHAPTER 7 Clinical Microbiology

Table 7–19 Differentiation of Aeromonas and Plesiomonas

<table>
<thead>
<tr>
<th>Organism</th>
<th>Esculin Hydrolysis</th>
<th>LDC</th>
<th>ODC</th>
<th>Mannitol</th>
<th>Arabinose</th>
<th>Sucrose</th>
<th>Inositol</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. hydrophila</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>A. caviae</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>A. sobria</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>P. shigelloides</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>+</td>
</tr>
</tbody>
</table>

LDC = lysine decarboxylase; ODC = ornithine decarboxylase; + = positive; − = negative

P. aeruginosa is also found to cause UTIs, endocarditis, and external otitis (swimmer’s ear). Infections may be difficult to control because of antibiotic resistance; this situation is usually only a problem in immunocompromised persons.

1. The growth requirements of P. aeruginosa are very simple. It has been found growing in distilled water. It also grows over a wide temperature range (4°C–42°C).

2. Identification of P. aeruginosa is not difficult and is summarized in Box 7–7. This organism grows well on most media and is easily recognized by its blue-green pigment (due to pyocyanin production) (see Web Color Image 7–68) and corn tortilla odor (some describe it as a grape-like odor). P. aeruginosa also produces the pyoverdin (see Web Color Image 7–69) that fluoresces under UV light and is oxidase-positive (see Web Color Image 7–38).

3. Other Pseudomonas species are of low virulence and rarely cause clinical disease. Many are environmental organisms. They can be opportunists, but are often considered as contaminants when isolated from clinical specimens.

a. P. fluorescens and P. putida are in the fluorescent Pseudomonas group, but are differentiated from P. aeruginosa as neither produces pyocyanin or grows at 42°C.

b. P. stutzeri is a rare pathogen, but may cause infection in an immunocompromised host. The organism demonstrates characteristic yellow to brown wrinkled, leathery, adherent colonies.

D. Infections by Burkholderia (Pseudomonas) cepacia are primarily nosocomial infections related to contaminated disinfectants used for antisepsis. Community-acquired infections are rare except in intravenous drug abusers. The bacterium has been associated with pneumonia in patients with cystic fibrosis.

E. Pathogenic Burkholderia species are known to cause severe infections, but are seldom seen in the United States. These species are considered by government agencies to be potential agents of bioterrorism.

1. Burkholderia mallei causes glanders, which is a zoonosis primarily affecting livestock, and can produce local suppurative or acute pulmonary infections in humans.

2. Burkholderia (Pseudomonas) pseudomallei causes melioidosis, an aggressive granulomatous pulmonary disease. Endemic areas include Southeast Asia, Northern Australia, and Mexico.

Table 7–20 Differentiation of Oxidase-Positive, Glucose-Fermentative, Gram-Negative Bacilli

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Aeromonas</th>
<th>Plesiomonas</th>
<th>Vibrio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Susceptible to O(129):</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 mg</td>
<td>−</td>
<td>+/−</td>
<td>+/−</td>
</tr>
<tr>
<td>150 mg</td>
<td>−</td>
<td>+/−</td>
<td>+/−</td>
</tr>
<tr>
<td>Ferment glucose</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Gelatin</td>
<td>+</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Growth on TCBS agar</td>
<td></td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>NaCl requirement</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Ferment inositol</td>
<td>−</td>
<td>−</td>
<td>−/−</td>
</tr>
</tbody>
</table>

TCBS = thiosulfate-citrate-bile-sucrose; O(129) = 2,4-diamino-6,7-diisopropylpteridine phosphate; + = positive; −/− = negative.
Box 7–6 Most Commonly Isolated Gram-Negative, Nonfermentative Rods

- Pseudomonas aeruginosa
- Burkholderia (Pseudomonas) cepacia
- Stenotrophomonas (Xanthomonas) maltophilia
- Acinetobacter
- Chryseobacterium (Flavobacterium) meningosepticum
- Eikenella corrodens
- Moraxella
- Alcaligenes and Achromobacter

F. **Stenotrophomonas (Xanthomonas) maltophilia** is found in the environment and causes a wide range of *nosocomial* infections.

G. **Acinetobacter** species are *opportunists* found in soil and water. They cause *pneumonia* and *UTIs*. On Gram’s stain, this genus is characteristically a *fat coccobacillus*. The two species most commonly seen in clinical specimens are *A. baumannii* (previously *A. calcoaceticus var. anitratus*) and *A. lwoffii* (previously *A. calcoaceticus var. lwoffii*).

H. **Chryseobacterium (Flavobacterium) meningosepticum** is a cause of neonatal meningitis or *septicemia*, especially in premature infants. The bacterium can cause pneumonia, endocarditis, bacteremia, and meningitis in adults.

I. **Eikenella corrodens** is found as *normal mouth and nasopharyngeal flora*. Trauma to the face or mouth, including dental work, may predispose an individual to infection. Human bite wounds are another source of infection. This isolate is often found as part of a mixed infection. The name is derived from the *corroding* or *pitting of the agar* by the colonies.

J. **Moraxella** species are *normal flora of mucous membranes*. They infrequently cause infection. *Conjunctivitis* is caused by *M. lacunata*. *M. catarrhalis* is *normal oral flora* of the respiratory tract and may cause *otitis media, sinusitis*, and *respiratory infections* (see Section X).

K. **Alcaligenes and Achromobacter** are divided into asaccharolytic and saccharolytic species. These opportunists are found in water and are resistant to disinfectants. The asaccharolytic *Alcaligenes faecalis* is most often seen in clinical specimens and has been isolated from urine, sputum, wound, and blood. *Achromobacter xylosoxidans subsp. xylosoxidans*, an oxidizer, has been associated with *otitis media, meningitis, pneumonia, surgical wound infections, UTIs, peritonitis*, and *bacteremia*.

L. The antibiotic susceptibility patterns of gram-negative nonfermentative bacilli are similar to those of the Enterobacteriaceae. Some nonfermenters are resistant to most of the antibiotics used.

Box 7–7 Identification of *Pseudomonas aeruginosa*

- Gram-negative rod
- Grows on most media
- Colonies have a feathered, ground (frosted) glass appearance
- β-hemolytic
- Corn tortilla odor (some prefer to describe it as a grapelike odor)
- Blue-green pigment (pyocyanin)
- Fluorescence upon exposure to ultraviolet light (pyoverdin)
- Grows between 4°C and 42°C
- Oxidase positive
- Oxidases
CHAPTER 7 Clinical Microbiology

Table 7–21 Identification of Nonfermentative, Gram-Negative Bacilli and Cocccobacilli

<table>
<thead>
<tr>
<th>Species</th>
<th>Oxidase</th>
<th>Catalase</th>
<th>Indole</th>
<th>Growth on MacConkey Agar</th>
<th>Motility</th>
<th>O/F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acinetobacter baumannii</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Acinetobacter lwoffi</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Burkholderia cepacia</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Stenotrophomonas maltophilia</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Flavobacterium meningosepticum</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Eikenella corrodens</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Moraxella species</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Achromobacter xylosoxidans</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
</tbody>
</table>

O/F = oxidation/fermentation; + = positive; − = negative.

The laboratory identification of the most common bacteria in this group is summarized in Table 7–21.

XV. MISCELLANEOUS GENERA

A. Legionella species are found worldwide in the environment. Most infections result from contaminated water sources. Legionella pneumophila is the most common isolate.

1. Pneumonia (Legionnaires’ disease) is the most common infection caused by Legionella. Pontiac fever is a nonpneumonic form of Legionella infection with flu-like symptoms. Its incidence in the general population is unknown.

2. Specimens for culture are generally from pulmonary sources.

3. Various staining methods can be used to visualize Legionella. The organisms are small gram-negative rods and stain weakly on Gram’s stain. Giemsa stain may be used. A direct fluorescent antibody test is available and is very useful for detection from direct specimens.

4. Culture can be accomplished using special media, because Legionella does not grow on blood agar. Cysteine is required for growth. The recommended medium for isolation is buffered charcoal yeast extract agar (BCYE). After several days of incubation, the colonies are gray-white to blue-green. Definitive identification can be made with fluorescent antibody or DNA probes.

B. Chromobacterium is characterized by a violet pigment. It is an opportunist that causes wound infections and bacteremia with a skin lesion as the typical portal of entry. Chromobacterium is found in soil and water, most commonly in tropical and subtropical climates. However, it has been found in the southeastern United States.

C. Gardnerella vaginalis is often associated with bacterial vaginosis. The organism is not thought to be the etiologic agent, but coinfections with the anaerobe Mobiluncus and other anaerobic bacteria are common. Human blood Tween agar is used for isolation with incubation at 48 hours in 5% to 10% carbon dioxide. Colonies are β hemolytic. Gram’s stain shows small gram-variable or gram-negative cocccobacilli. A presumptive identification can be made when clue cells, squamous epithelial cells covered with tiny bacilli, are observed in wet mounts or direct smears of vaginal discharge.

D. Streptobacillus moniliformis is the agent of rat-bite fever (streptobacillosis), an infection characterized by fever, flu-like symptoms, a macropapular rash, and lymph node
involvement. Gram’s stain shows pleomorphic, long filamentous forms. In blood culture media, the organism grows as “fluff balls” or “bread crumbs.” Serum antibodies can be detected by agglutination tests.

E. *Spirillum minus* (minor) is also the agent of rat-bite fever (spirillosis). Symptoms of infection are the same as those for streptobacillosis. Serum antibodies can also be detected by agglutination tests. Penicillin G is the treatment of choice for both infections.

F. *Bartonella bacilliformis* is the etiologic agent of bartonellosis, also known as verruca peruana. Geographically, the disease is restricted to the high-altitude valleys of Peru, Ecuador, and southwest Columbia. The febrile systemic infection is transmitted to humans by the bite of infected sandflies. The organism is an aerobic, pleomorphic, poorly staining, gram-negative bacterium.

XVI. MYCOPLASMA, UREAPLASMA, AND THE CHLAMYDIACEAE

A. The organisms in the family Mycoplasmataceae are small, free-living organisms that lack a cell wall. The three most common isolates are *Mycoplasma pneumoniae*, *Mycoplasma hominis*, and *Ureaplasma urealyticum*.

1. *M. pneumoniae* is the causative agent of atypical or walking pneumonia. This organism is not normal flora, and isolation is always significant.

2. *U. urealyticum* and *M. hominis* colonize the lower genitourinary tracts of up to 60% to 70% of the normal population. They cause opportunistic infections such as nongonococcal urethritis in males and postpartum infections in females.

3. The organisms can be cultured on special enriched media. *Mycoplasma* colonies are very small, requiring a dissecting microscope for observation. They demonstrate a characteristic “fried egg” appearance (raised center with flat edges). Colonies of *Ureaplasma* are much smaller.

4. Dienes (see Web Color Image 7–70) or methylene blue stains can be used to visualize the colonies on agar media. Immunofluorescent stains and serology testing are used for laboratory diagnosis. Differentiation of colonization and infection may be difficult in test results positive for *U. urealyticum* or *M. hominis*, because both can be normal flora.

B. Members of the family Chlamydiaceae are obligate intracellular parasites. The three most common species are *Chlamydia trachomatis*, *Chlamydophila pneumoniae* (previously *Chlamydia pneumoniae*), and *Chlamydophila psittaci* (previously *Chlamydia psittaci*). There are two forms in the growth cycle of the organism: the *elementary body* is the infectious form, and the noninfectious *reticulate body* is the intracellular reproductive form.

1. *C. trachomatis* is the causative agent of trachoma, lymphogranuloma venereum, and various other sexually transmitted diseases. Trachoma is a leading cause of blindness worldwide. *C. trachomatis* is the most common sexually transmitted bacterial pathogen in the United States.

2. *C. pneumoniae* is an important cause of pneumonia and pharyngitis.

3. *C. psittaci* is the cause of psittacosis (ornithosis or parrot fever), a respiratory tract disease seen in patients exposed to birds.

4. Laboratory diagnosis can be accomplished several ways. *Shell vial* tissue cultures are used for detection of *Chlamydia*. Cell lines, usually McCoy cells, can be inoculated with specimens suspected of harboring *Chlamydia*. After 72 hours, staining with iodine shows darkly stained inclusion bodies within the cells. Fluorescein-labeled monoclonal antibodies can also be used to detect the chlamydial inclusions in Shell vial cultures. Other methods of laboratory diagnosis available include immunofluorescence, enzyme immunoassay, nucleic acid probes, and polymerase chain reaction.
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XVII. SPIROCHETES

A. Three genera in the order Spirochaetales cause human disease: *Treponema*, *Leptospira*, and *Borrelia*. These bacteria are characterized by their helical shape with spiral coils (see Web Color Image 7–71). The pathogenic spirochetes and associated infections are listed in Table 7–22.

B. *Treponema pallidum* ssp. *pallidum* is the etiologic agent of syphilis. The infection progresses through three phases: primary, secondary, and tertiary. Skin lesions called chancres are characteristic of primary and secondary syphilis. Chancres are extremely infectious and fluid from these lesions can be used to perform darkfield microscopy. The observation of motile, spiral-shaped treponemes from the chancre in the primary and secondary stages of syphilis is diagnostic. Diagnosis is usually done by serologic testing, which requires either a nonspecific or specific treponemal test.

1. The nonspecific tests include the rapid plasma reagin (RPR) and venereal disease research laboratory tests (VDRL).
2. Treponemal tests detect antibodies specific for treponemal antigens. These tests include the fluorescent treponemal antibody absorption test (FTA-ABS) and the TP-PA test, a modification of the MHA-TP test (microhemagglutination test for *T. pallidum*)..

C. *Treponema* species are the etiologic agents of nonvenereal treponemal diseases. These diseases are endemic to developing countries, humid tropical areas, or arid dry areas. They are not found in the United States. Transmission is via direct contact, person-to-person, or sharing contaminated eating utensils. Skin lesions, gummas, and dissemination are characteristic clinical manifestations.

1. *Treponema pallidum* ssp. *pertenue* is the etiologic agent of Yaws.
2. *Treponema pallidum* ssp. *endemicum* is the etiologic agent of Bejel or endemic syphilis.
3. *Treponema carateum* is the etiologic agent of Pinta.

D. *Borrelia* is the cause of relapsing fever (*B. recurrentis*) and Lyme disease (*B. burgdorferi*), both of which are spread by ticks. Relapsing fever is also associated with louse-borne transmission. Erythema chronicum migrans (EMC) is associated with Lyme disease, and is characterized by classic skin lesions at the site of the tick bite. Relapsing febrile episodes are caused by antigenic variation in the spirochete and are characteristic of infections caused by *B. recurrentis*. Laboratory diagnosis of relapsing fever is achieved

### Table 7–22 Spirochetes and Associated Infections

<table>
<thead>
<tr>
<th>Spirochete</th>
<th>Clinical Infection</th>
<th>Transmission</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Treponema pallidum</em> ssp. <em>pallidum</em></td>
<td>Syphilis</td>
<td>Sexual contact; nongenital contact; transplacental transmission</td>
</tr>
<tr>
<td><em>T. pallidum</em> ssp. <em>pertenue</em></td>
<td>Yaws</td>
<td>Direct contact</td>
</tr>
<tr>
<td><em>T. pallidum</em> ssp. <em>endemicum</em></td>
<td>Bejel (endemic syphilis)</td>
<td>Direct contact; sharing contaminated eating utensils</td>
</tr>
<tr>
<td><em>T. carateum</em></td>
<td>Pinta</td>
<td>Person-to-person contact; rare sexual transmission</td>
</tr>
<tr>
<td><em>Borrelia recurrentis</em></td>
<td>Relapsing fever–endemic and epidemic</td>
<td>Ticks and lice; Epidemic: Ornithodoros ticks Epidemic: Pediculus humanus louse</td>
</tr>
<tr>
<td><em>B. burgdorferi</em></td>
<td>Lyme disease</td>
<td>Ixodes tick</td>
</tr>
<tr>
<td><em>Leptospira interrogans</em></td>
<td>Leptospirosis (Weil’s disease)</td>
<td>Animal reservoirs–dogs, rats, rhodentic Contact with urine of carriers; contaminated bodies of water</td>
</tr>
</tbody>
</table>
by observation of spirochetes in blood smears stained with the Giemsa or Wright stain. Diagnosis of lyme disease is determined by serologic means. Antibody in the patient’s serum may be detected after the third week of the illness. Sensitivity of the various methods varies widely from poor to good.

E. *Leptospira interrogans* is the cause of leptospirosis or Weil’s disease. Animals reservoirs are dogs, rats, and rodents. The organism is transmitted via exposure to the urine of carriers or urine-contaminated soil or water. The organism can be cultured using Fletcher medium, a semi-solid, tubed medium. Growth occurs in a ring just beneath the medium surface. Stuart liquid media can also be used for culture. Diagnosis may be made from microscopy (dark-field, phase-contrast, or immunofluorescent microscopy) or culture. Serological diagnosis, most commonly used, is via slide agglutination or IgM dot-ELISA.

## XVIII. MYCOBACTERIA

A. The mycobacteria are aerobic bacilli. The cell wall is rich in lipids, which makes them resistant to Gram’s stain. After staining, the bacilli are difficult to decolorize with acid solutions (e.g., they are acid-fast). Most members of this group grow slowly with cellular division every 12 to 24 hours. Identification of a *Mycobacterium* species isolate is determined by the characteristics outlined in Box 7–8.

B. The most common specimen collected and processed is respiratory secretions, although mycobacteria can be recovered from virtually any body site. With sputum and other specimens collected from nonsterile sites, digestion and decontamination must be done before inoculation of growth media. See Box 7–9 for digestion and decontamination agents.

C. Following digestion and decontamination, the suspension is diluted with buffer and centrifuged to concentrate any organisms present. The sediment is inoculated onto mycobacterial growth media and used to make a smear for acid-fast staining.

1. Mycobacterial growth media
   a. The most commonly used egg-based medium is *Löwenstein-Jensen*.
   b. Agar-based media are variations of Middlebrook 7H10 medium. All contain some inhibitory agents to suppress the growth of contaminating bacteria.

2. Acid-fast stains
   a. Carbolfuchsin stains include the *Ziehl-Neelsen* and *Kinyoun*, which use carbolfuchsin as the primary stain, acid alcohol for the decolorizing agent, and a methylene blue counterstain. The *Ziehl-Neelsen* stain utilizes heat to drive the stain into the mycobacterial cell wall. The *Kinyoun* stain is a “cold” variation of

### Box 7–8 Characteristics Used to Identify *Mycobacterium* Isolates

- Acid fast
- Colony morphology
- Colony pigmentation and photoreactivity
- Growth rate
- Growth temperature
- Biochemical tests

### Box 7–9 Decontamination and Digestion Agents

- Sodium hydroxide
- N-acetyl-L-cysteine
- Benzalkonium chloride
- Oxalic acid
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Table 7–23 Characteristics of Mycobacterial Photoreactivity Groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Pigment Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Photochromogens</td>
<td>Produce pigment on exposure to light</td>
</tr>
<tr>
<td>Scotochromeens</td>
<td>Produce pigment in the light or dark</td>
</tr>
<tr>
<td>Nonchromogens</td>
<td>No pigment produced in the light or dark</td>
</tr>
</tbody>
</table>

The stain. Stained smears are viewed with a light microscope. Acid-fast organisms stain pink or red against a blue background. Nonacid-fast organisms stain blue (see Web Color Image 7–8).

b. The fluorochrome stains—auramine and auramine-rhodamine—are very sensitive and require a fluorescence microscope. Acid-fast organisms fluoresce a yellow-orange against a dark background (see Web Color Image 7–9).

D. Pigment groups are helpful in a presumptive identification of the mycobacteria. The Runyon classification system was designed to classify the species within the genus. Three groups are based on photosensitivity: photochromogens (Group I), scotochromogens (Group II), and nonphotochromogens (Group III). The “rapid growers” are Group IV. The species within the MTB group, although nonchromogens, are categorized separately. The Mycobacterium species are still categorized according to photosensitivity, but the designation as “Runyon groups” is not often noted.

1. To determine pigment groups, or photoreactivity, a specimen is inoculated to two tubes or plates of mycobacterial media. One tube is incubated in the light, and the other is incubated in the dark. Following growth, pigment production of each is noted. The medium initially incubated in the dark is incubated in bright light for several hours, and pigment production, if any, is noted.

2. The nonphotochromogenic (nonchromogenic) mycobacteria do not produce pigment in either light or dark. Photochromogens produce pigment in light, whereas scotochromogens produce pigment in the light or dark. Pigment color may range from light yellow to a dark orange (see Web Color Images 7–72 and 7–73). The mycobacterial photoreactivity groups are summarized in Table 7–23.

E. The growth rate is determined as rapid or slow. Rapid growers produce colonies in fewer than 7 days. Slow growers require longer than 7 days for production of colonies. Table 7–24 lists some of the medically significant mycobacterial species arranged according to pigment groups and growth rate.

F. The optimal growth temperature for most Mycobacterium species is 35°C to 37°C. M. marinum grows best at 30°C, whereas M. xenopi grows best at 42°C.

Table 7–24 Medically Important Mycobacteria According to Pigment Group and Growth Rate

<table>
<thead>
<tr>
<th>Group</th>
<th>Organism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Photochromogen</td>
<td>Mycobacterium kansasii</td>
</tr>
<tr>
<td></td>
<td>M. marinum</td>
</tr>
<tr>
<td></td>
<td>M. simiae</td>
</tr>
<tr>
<td>Scotochromeen</td>
<td>M. gordonae</td>
</tr>
<tr>
<td></td>
<td>M. avium—M. intracellulare</td>
</tr>
<tr>
<td></td>
<td>M. scrofulaceum</td>
</tr>
<tr>
<td></td>
<td>M. szulgai</td>
</tr>
<tr>
<td></td>
<td>M. xenopi (N/S)</td>
</tr>
<tr>
<td></td>
<td>M. flavescens</td>
</tr>
<tr>
<td>Nonchromogen</td>
<td>M. tuberculosis</td>
</tr>
<tr>
<td></td>
<td>M. hovis</td>
</tr>
<tr>
<td></td>
<td>M. avium—M. intracellulare*</td>
</tr>
<tr>
<td>Rapid growers</td>
<td>M. chelonei</td>
</tr>
<tr>
<td></td>
<td>M. fortuitum</td>
</tr>
</tbody>
</table>

N = nonchromogen; S = scotochromeen.
*Some isolates (15%) are pigmented.
A Concise Review of Clinical Laboratory Science

### Table 7–25 Identification of Mycobacteria

<table>
<thead>
<tr>
<th>Organism</th>
<th>Photo Reactivity</th>
<th>Niacin Reactivity</th>
<th>Susceptible to TCH</th>
<th>Nitrate Reduction</th>
<th>SQ Catalase</th>
<th>Tween Hydrolysis</th>
<th>Growth in 5% NaCl</th>
<th>Iron Uptake</th>
<th>Arylsulfatase</th>
<th>Urease</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. marinum</td>
<td>Scotochromogen</td>
<td>V</td>
<td>−−</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>M. kansasii</td>
<td>Scotochromogen</td>
<td>V</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>M. simiae</td>
<td>Nonchromogen</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>M. avium—M.</td>
<td>Nonchromogen</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>intracellulare</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M. gordonae</td>
<td>Scotochromogen</td>
<td>−</td>
<td>−</td>
<td>++</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>V</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>M. scrofulaceum</td>
<td>Scotochromogen</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>V</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>M. szulgai</td>
<td>Scotochromogen</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>V</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>M. xenopi</td>
<td>Scotochromogen</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>V</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>M. flavescens</td>
<td>Scotochromogen</td>
<td>−</td>
<td>−</td>
<td>++</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>M. fortuitum</td>
<td>Rapid grower</td>
<td>−</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>V</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>M. chelonei</td>
<td></td>
<td></td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>V</td>
<td>−</td>
<td>++</td>
</tr>
</tbody>
</table>

SQ = semi-quantitative; TCH = thiophen-2-carboxylic acid hydrazide; V = variable; + = positive; − = negative.

G. There are several biochemical tests that are valuable in identification of the mycobacteria (Table 7–25). Most of these tests are performed only in those laboratories that do complete identification. Some tests, such as niacin and nitrate tests, are easy to perform and can give presumptive identification of *M. tuberculosis.*

1. **Niacin accumulation** is detected by measuring nicotinic acid, which reacts with cyanogen bromide in the presence of aniline to form a yellow compound.

2. **Nitrate reduction** is performed as with the method used for Enterobacteriaceae. A positive test is a red pigment.

3. All mycobacteria are **catalase-positive** (see Web Color Image 7–11). The quantity of catalase and production of **heat-stable catalase** are species-specific. **Semi-quantitative catalase production** (see Web Color Image 7–74) is determined by measuring the height of the column of bubbles when hydrogen peroxide and Tween 80 are added to a deep with mycobacterial growth. Catalase heat stability is determined by heating the specimen to 68°C for 20 minutes prior to the addition of hydrogen peroxide. Production of bubbles is a positive reaction.

4. **Tween hydrolysis** measures the presence of a lipase. Hydrolysis causes a pink color change.

5. **NaCl tolerance** is determined by inoculating an egg-based media with 5% NaCl and observing growth or no growth following incubation.

6. **Iron uptake** is determined by adding ferric ammonium citrate to the mycobacterial colonies. Dusty-brown colonies are a positive reaction for iron uptake and the formation of iron oxide.

7. **Arylsulfatase activity** is detected by adding phenolphthalein to the colony/substrate mixture and observing the formation of a pink color.

8. The **urease** test detects an organism’s ability to produce urease and hydrolyze urea to form ammonia, which produces an alkaline reaction. The increase in pH is detected by a change of color to pink or red.

9. **Susceptibility to thiophen-2-carboxylic acid hydrazide (TCH)** is determined by observing growth or no growth of mycobacteria following incubation with this compound.

10. **Nucleic acid amplification** is available for identification of some species of mycobacteria, including MTB complex and MAC complex. Specificity is very good, but use with clinical specimens may give false-negative results. Identification with this technique is usually performed in reference laboratories.

11. **Gas-liquid chromatography** and **high-performance liquid chromatography** are methods to analyze mycobacterial lipids.

H. *Mycobacterium tuberculosis* is a member of the MTB complex. MTB causes tuberculosis (TB), a pulmonary infection transmitted by the inhalation of droplet nuclei. *M. bovis* causes tuberculosis in humans, cattle, and other animals.
1. **Primary tuberculosis** is the initial infection. The *mycobacterium* is eradicated by the host cellular immune response or walled off in a granuloma in the lung. Reactivation of latent infections can occur in immunocompromised individuals and cause secondary tuberculosis. Miliary TB is a disseminated infection with multiple organ involvement. Pott’s disease is military tuberculosis in the bones or spine.

2. The **tuberculin skin test** is used to detect MTB-infected individuals. A purified protein derivative (PPD) is the MTB antigen. A hypersensitivity reaction at the injection site within 72 hours is a positive test. A positive skin test does not distinguish patients with active disease from those with latent infections.

3. **Identifying characteristics of *M. tuberculosis***: slow growth, nonchromogenic, “serpentine cording,” niacin accumulation-positive, nitrate-positive, and susceptible to NAP. Web Color Image 7–75 depicts a Kinyoun acid-fast stain with the characteristic cell aggregates of *M. tuberculosis*, due to the cording factor.

**I. Mycobacterium avium complex (MAC)** consists of the environmental organisms *M. avium* and *M. intracellulare*. It may colonize healthy individuals or cause opportunistic infections in immunocompromised individuals.

1. It is most commonly associated with disseminated disease in AIDS patients.
2. Identifying characteristics of MAC: slow growth, semiquantitative catalase >45 mm, and most are tellurite-positive. Otherwise, it is relatively inactive biochemically.

**J. MOTT. Mycobacterium other than tuberculosis** include several species that are environmental organisms that may colonize individuals or cause a variety of opportunistic infections. They are classified according to growth rate and photosensitivity. The mycobacteria are also known as “atypical mycobacteria” or NTM, mycobacteria other than the tuberculous mycobacteria.

**K. Mycobacterium leprae** is the etiologic agent of leprosy or Hansen’s disease. The mycobacterium cannot be cultured in vitro, but can be grown in mouse footpads or the armadillo.

**L. Identifying characteristics** of select **Mycobacterium** species are summarized in Table 7–25. Infections caused by mycobacteria are summarized in Table 7–26.

### XIX. ANAEROBES

**A. Atmospheric requirements.** Anaerobes constitute the majority of bacteria found in and on the human host. **Obligate anaerobes** grow in the absence of oxygen and vary in their
tolerance to oxygen. Oxygen and its derivatives (e.g., hydrogen peroxide) are toxic. Most require a low redox potential, because an environment with a high redox potential is oxidized and harmful to anaerobic bacteria. These bacteria can be divided into moderate and strict anaerobes. The majority of medically significant anaerobes are moderate anaerobes.

1. Moderate obligate anaerobes can tolerate an atmosphere containing low levels of oxygen (2%–8%).
2. Strict obligate anaerobes are killed by exposure to oxygen. They cannot tolerate more than 0.5% oxygen.
3. Aerotolerant anaerobes grow very poorly in ambient air (21% oxygen), but grow well under anaerobic conditions.
4. Facultative anaerobes can grow aerobically and anaerobically.

B. Direct examination of a specimen using Gram’s stain is very helpful. Typical anaerobe morphology (pleomorphic rods; gram-positive, boxcar-shaped cells; thin rods with pointed ends) may be evident. Also, a positive Gram’s stain with negative aerobic culture results may indicate the need for anaerobic culture. If aerobic and anaerobic cultures have been performed, negative results may indicate a problem with collection, transport, or culture conditions. The Gram’s stain can also determine the culture conditions and media to be used.

A list of the commonly isolated anaerobes with their gram reactions are listed in Table 7–27.

Table 7–28 summarizes the microscopic morphology of the more common anaerobes.

C. The media that are used to grow and identify anaerobes are as follows. Anaerobic bacteria grow best on pre-reduced, anaerobically sterilized media (PRAS) that are sterilized and stored under anaerobic conditions. Culture media may be enriched with hemin and vitamin K.

1. Anaerobic sheep blood agar is a general growth medium for all anaerobes. It is supplemented with vitamin K and hemin, and the type of agar base may vary (e.g., Columbia, Schaedler, brain-heart infusion). CDC blood agar and Brucella blood agar (BRU-BA) are most commonly used as all-purpose, direct plating media.

### Table 7–27 Gram Reaction of Anaerobes

<table>
<thead>
<tr>
<th>Organism</th>
<th>Gram Negative</th>
<th>Gram Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacilli</td>
<td>Cocci</td>
<td>Bacilli</td>
</tr>
<tr>
<td>Bacteroides</td>
<td>Veillonella</td>
<td>Actinomyces</td>
</tr>
<tr>
<td>Fusobacterium</td>
<td></td>
<td>Bifidobacterium</td>
</tr>
<tr>
<td>Porphyromonas</td>
<td>Clostridium</td>
<td></td>
</tr>
<tr>
<td>Prevotella</td>
<td>Eubacterium</td>
<td></td>
</tr>
<tr>
<td>Porphyromonas</td>
<td>Lactobacillus</td>
<td></td>
</tr>
<tr>
<td>Prevotella</td>
<td></td>
<td>Mobiluncis</td>
</tr>
<tr>
<td>Peptostreptococcus</td>
<td></td>
<td>Propionibacterium</td>
</tr>
<tr>
<td>Bifidobacterium</td>
<td>Meleagris</td>
<td></td>
</tr>
<tr>
<td>Propionibacterium</td>
<td>Pleomorphic, diphtheroid</td>
<td></td>
</tr>
<tr>
<td>Eubacterium</td>
<td>Pleomorphic, diphtheroid</td>
<td></td>
</tr>
<tr>
<td>Peptostreptococcus</td>
<td>Pairs and chains</td>
<td></td>
</tr>
<tr>
<td>Veillonella</td>
<td>Clumps and short chains</td>
<td></td>
</tr>
</tbody>
</table>

### Table 7–28 Microscopic Morphology of Common Anaerobes

<table>
<thead>
<tr>
<th>Organism</th>
<th>Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prevotella melaninogenica</td>
<td>Cocccobacilli</td>
</tr>
<tr>
<td>Bacteroides fragilis</td>
<td>Pale-staining rods</td>
</tr>
<tr>
<td>Fusobacterium necrophorum</td>
<td>Pleomorphic rods with round to tapered ends</td>
</tr>
<tr>
<td>F. nucleatum</td>
<td>Long, slender with sharply pointed or tapered ends</td>
</tr>
<tr>
<td>Clostridum</td>
<td>“Boxcar” rods with blunt ends</td>
</tr>
<tr>
<td>Actinomyces</td>
<td>Branching, thin filamentous rods</td>
</tr>
<tr>
<td>Bifidobacterium</td>
<td>Diphtheroid; cocccoid or thin pointed; may be branching</td>
</tr>
<tr>
<td>Propionibacterium</td>
<td>Pleomorphic; diphtheroid</td>
</tr>
<tr>
<td>Eubacterium</td>
<td>Pleomorphic; diphtheroid</td>
</tr>
<tr>
<td>Peptostreptococcus</td>
<td>Pairs and chains</td>
</tr>
<tr>
<td>Veillonella</td>
<td>Clumps and short chains</td>
</tr>
</tbody>
</table>
2. **Bacteroides bile esculin (BBE) agar** is used for the selection and presumptive identification of *Bacteroides fragilis* group. The high concentration of bile is inhibitory to other organisms. Colonies of the *B. fragilis* group hydrolyze esculin and appear black, surrounded by a black halo (see Web Color Image 7–76).

3. **Phenylethyl alcohol blood agar (anaPEA)** inhibits the gram-negative facultative bacilli, whereas most anaerobes grow well on the medium.

4. **Kanamycin-vancomycin laked blood agar (KVLB)** selects for *Prevotella* and *Bacteroides*. Other gram-positive and gram-negative rods are inhibited by the kanamycin and vancomycin. Laked blood enhances production of a brown-black pigment by certain *Prevotella* species.

5. **Egg-yolk agar** is used to determine if an isolate produces lecithinase or lipase. The lecithin in egg yolk is split by lecithinase, which results in an opaque halo around the colony (see Web Color Image 7–77). Lipase degrades triglycerides into glycerol and free fatty acids, and is detected by observing an oily or “mother-of-pearl” sheen on the colony surface (see Web Color Image 7–78). Protelysis results in a small clear halo around the colonial growth.

D. Tests used for the identification of anaerobes are as follows.

1. The **catalase** test for anaerobes uses 15% hydrogen peroxide instead of 3%.
2. A **spot indole test** is easily and quickly performed. This test uses p-dimethylaminocinnamaldehyde as the developing reagent. A blue color is produced when the test is positive (see Web Color Image 7–80).
3. Special potency **antibiotic disks** can be very helpful in the identification of anaerobic bacteria. The susceptibility of anaerobes to colistin (10 mcg), vancomycin (5 mcg), and kanamycin (1 mg) varies. Most gram-negative anaerobes are resistant to vancomycin, while most gram-positive anaerobes are susceptible to vancomycin and resistant to colistin. Gram-negative anaerobes vary in their susceptibility to colistin.
4. Growth in 20% bile separates the bile-resistant *B. fragilis* group from *Prevotella*. Bile disks, broth, or bile-containing agar can be used to perform the test. Escluin hydrolysis (see Web Color Image 7–76) is often detected with the same agar, and the reaction may also be helpful in the identification of anaerobic gram-negative rods.
5. Susceptibility to a 1-mg sodium polyanethol sulfonate (SPS) disk is characteristic of *Peptostreptococcus anaerobius*.
6. The **nitrate test** is performed with a disk placed on an inoculated anaBAP with the test organism. Following incubation, sulfanilic acid, dimethyl-α-naphthylamine, and zinc (if necessary) are added to the disk. The test is interpreted in the same manner as the tube test.
7. A reverse **CAMP test** identifies *Clostridium perfringens*. In this test, the *Clostridium* isolate is streaked perpendicular to a known group B *Streptococcus agalactiae*. A positive test demonstrates characteristic arrowhead hemolysis.
8. The **Nagler Test** is a laboratory test for the detection of lecithinase. This test uses *C. perfringens* type A antitoxin to neutralize lecithinase. The antitoxin inhibits the lecithinase reaction on egg-yolk agar. The test has been used for the presumptive identification of *C. perfringens*. However, it is not used as frequently, because other *Clostridium* species are now known to be Nagler test-positive.
9. Lecithinase, lipase, and proteolysis are all detected on **egg-yolk agar** (Color Images 7–77 and 7–78). Characteristic reactions are described above in the anaerobic media section.

E. Alternate methods may be used for the identification of anaerobic bacteria.

1. **Gas-liquid chromatography (GLC)** is used to detect metabolic end products (e.g., propionic acid, lactic acid) or cellular fatty acids (membrane components). Certain anaerobic bacteria have a characteristic GLC pattern.
Table 7–29 Identification of Clinically Encountered Anaerobic Gram-Negative Bacilli

<table>
<thead>
<tr>
<th>Species</th>
<th>Vancomycin</th>
<th>Kanamycin</th>
<th>Colistin</th>
<th>Indole</th>
<th>Lipase</th>
<th>Esculin</th>
<th>Hydrolysis</th>
<th>Growth in 20% Bile</th>
<th>Catalase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteroides fragilis</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>B. thetaiotaomicron</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>B. vulgatus</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>B. ureolyticus</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Prevotella intermedia</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>P. melaninogenica</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Porphyromonas anaerobacteriana</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Fusobacterium nucleatum</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>F. necrophorum</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>F. mortiferum</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
</tbody>
</table>

R = resistant; S = susceptible; + = positive; – = negative.

2. Conventional biochemical tubes (“roll-tubes”) are the “gold standard” and are performed in reference laboratories. PRAS media supplemented with substrates is used for biochemical testing.

3. Commercial identification systems are miniaturized biochemical test panels or rapid enzymatic systems that detect preformed enzymes.

F. The characteristics of the more commonly isolated anaerobes, including the types of infections caused by each, are as follows.

1. The gram-negative bacilli cause a wide variety of infections. These organisms are part of the normal flora in the gastrointestinal tract, the female genital tract, and the oropharynx. Key characteristics of the most common species are summarized in Table 7–29.

   a. Members of the Bacteroides fragilis group are bile-resistant, and therefore grow in broth with 20% bile. Growth occurs on BB6 agar with characteristic black colonies, because the organisms also hydrolyze esculin (see Web Color Image 7–76) with few exceptions. This group of anaerobic gram-negative rods is resistant to vancomycin, kanamycin, and colistin.

   (1) B. fragilis is the most common anaerobic isolate. In addition to the above characteristics, it is indole-negative and catalase-positive.

   (2) B. vulgatus, a member of the B. fragilis group, is indole-negative, catalase-positive, and esculin-negative.

   (3) Presumptive identification of B. thetaiotaomicron is based on positive indole and catalase reactions.

   b. The Bacteroides ureolyticus group and Bilophila wadsworthia are differentiated from the B. fragilis group in that they are nitrate-positive, vancomycin-resistant, kanamycin-susceptible, and colistin-susceptible.

   c. Prevotella and Porphyromonas are bile sensitive. Most of the clinically important species are pigmented dark brown or black (see Web Color Image 7–81). Laked blood agar enhances pigment production by these anaerobes. Both demonstrate a brick-red fluorescence when exposed to ultraviolet light.

   d. Porphyromonas species are susceptible to vancomycin, whereas other gram-negative anaerobic bacilli are vancomycin-resistant. Porphyromonas species will not grow on kanamycin-vancomycin laked blood agar (KVLB). They require hemin and vitamin K for growth.

   e. Fusobacterium nucleatum and F. necrophorum are normal flora in the respiratory and gastrointestinal tracts. F. nucleatum is a long, slender, gram-negative rod with tapered ends (see Web Color Image 7–82) and is indole-positive and lipase-negative. F. necrophorum is a pleomorphic gram-negative rod that is indole-positive and lipase-positive. Both demonstrate chartreuse fluorescence when exposed to ultraviolet light.
2. The gram-positive bacilli are found as normal flora and are also widely distributed in the environment (e.g., *Clostridium* species).
   a. *Clostridium* species (Table 7–30) cause a wide variety of infections. These gram-positive bacilli produce endospores that survive in adverse environmental conditions and germinate when conditions are favorable for bacterial growth.
      (1) *C. botulinum* is the etiologic agent of botulism. The botulin neurotoxin is the most potent toxin known.
      (2) *C. tetani* causes tetanus (lockjaw). It is characterized by swarming colonies and rods with round, terminal spores and a “drumstick” or “tennis racket” appearance (see Web Color Image 7–83). The clinical laboratory has little role in the diagnosis of either *C. botulinum* or *C. tetani*. The diagnosis is based on the clinical symptoms.
      (3) *C. difficile* is associated with antibiotic-associated diarrhea and pseudomembranous enterocolitis.
         (a) Treatment with broad-spectrum antibiotics may suppress the normal intestinal flora.
         (b) *C. difficile*, which produces a toxin, is able to proliferate, resulting in diarrhea or colitis.
         (c) Tests to detect *C. difficile* toxin are available (e.g., tissue culture, latex agglutination, enzyme-immunoassay), and may be performed on bacterial isolates or stool specimens. Toxicity tests should be performed, because not all strains are toxigenic.
         (d) *C. difficile* has a characteristic “horse-stable” odor, produces yellow, ground-glass colonies on CCFA agar (see Web Color Image 7–79), and demonstrates a chartreuse fluorescence upon exposure to ultraviolet light.
      (4) Gas gangrene (myonecrosis) can be caused by *C. perfringens*, *C. septicum*, *C. sporogenes*, and *C. novyi*. Clostridial endospores are introduced into tissue by trauma or surgery. The spores germinate in vivo, and the organisms produce gas and cause extensive muscle and tissue necrosis.
         (a) *C. perfringens* demonstrates a characteristic double-zone hemolysis (see Web Color Image 7–84) on anaerobic blood agar, is Nagler test-positive, lecithinase-positive, and reverse Camp test-positive. Presumptive identification of *C. perfringens* is summarized in Table 7–31.
         (b) *C. septicum* is nonhemolytic and its colonies swarm and have a characteristic "medusa-head" appearance. It is lecithinase-negative and hydrolyzes esculin.
   b. The gram-positive, nonspore-forming bacilli are normal flora in various body sites. Their significance of infections is secondary when compared with infections caused by the *Clostridium*.

### Table 7–30 Identification of *Clostridium* Species

<table>
<thead>
<tr>
<th>Organism</th>
<th>Lecithinase</th>
<th>Lipase</th>
<th>Esculin Hydrolysis</th>
<th>Double Zone Hemolysis</th>
<th>Reverse Camp</th>
<th>Motility</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. perfringens</em></td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td><em>C. novyi</em></td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td><em>C. septicum</em></td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td><em>C. sporogenes</em></td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
</tbody>
</table>

CAMP = Christie, Atkins, and Munch-Petersen; + = positive; − = negative.

### Table 7–31 Presumptive Identification of Anaerobic Gram-Positive Bacilli

<table>
<thead>
<tr>
<th>Organisms</th>
<th>Spores</th>
<th>Kanamycin</th>
<th>Vancomycin</th>
<th>Collistin</th>
<th>Indole</th>
<th>Catalase</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Clostridium perfringens</em></td>
<td>+</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td><em>Propionibacterium acnes</em></td>
<td>−</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>Eubacterium lentum</em></td>
<td>−</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

R = resistant; S = susceptible; + = positive; − = negative.
(1) **Actinomyces israelii** is the most frequently isolated member of the anaerobic genus *Actinomyces*. Infections often involve periodontal disease. **Sulfur granules** may be present in exudate. The gram-positive rods have a pleomorphic, beaded and branching appearance (see Web Color Image 7–85), and older colonies have a characteristic “molar-tooth” morphology (see Web Color Image 7–86).

(2) **Bifidobacterium** species are part of the normal intestinal flora. The rods are pleomorphic and may have two forks. Identification is difficult unless laboratory personnel are experienced with anaerobes.

(3) **Eubacterium** species are normal intestinal and oral flora and also are difficult to identify. *E. lentum* is a pleomorphic gram-positive rod and is nitrate-positive and catalase-negative. Characteristics used for presumptive identification are summarized in Table 7–31.

(4) **Propionibacterium** are common on skin. *P. acnes* is the most common isolate. This organism is seen as a contaminant in much the same fashion as coagulase-negative staphylococci. The propionibacteria are often referred to as “anaerobic diphtheroids.” A pleomorphic, gram-positive rod that is catalase and indole-positive is presumptively identified as *P. acnes* (Table 7–31).

(5) **Lactobacillus** species may be facultative or obligate anaerobes. They are normal flora of the mouth, intestinal tract, and vagina. The lactobacilli are long chains of gram-positive rods and are catalase-negative.

(6) **Mobiluncus** species are curved gram-positive rods that stain gram-variable. They are associated with bacterial vaginosis, a poly-microbial infection. Microscopic observation of “clue cells” in vaginal exudates may be indicative of an infection. *Gardnerella vaginalis* is often associated with *Mobiluncus* and bacterial vaginosis.

### XX. ZOONOTIC AND RICKETTSIAL INFECTIONS

**A. Zoonotic infections** are diseases of animals that infect humans who have contact with infected animals. Animals are the natural hosts, and transmission to humans may be via inhalation, contact with animal secretions, carcasses or products, animal bites and scratches, or by arthropod vectors. Refer to Table 7–32 for a list of bacterial agents and associated with zoonotic infections. The natural host, vector, mode of transmission and associated infections are indicated for each bacterium.

**B. Members of the Family Rickettsiaceae are obligate intracellular pathogens** and causative agents of arthropod-borne human infections (Table 7–33). Animals or rodents are the natural hosts, and transmission to humans is usually through arthropod vectors. Refer to Table 7–33 for a list of bacterial agents and associated infections are indicated for each bacterium.

1. **Rocky mountain spotted fever** is transmitted by the tick and is caused by *R. rickettsii*. Humans are accidental hosts.

2. **Endemic typhus**, also called *murine typhus*, is transmitted by rat fleas and caused by *R. typhi*. Rats are reservoirs, whereas humans and other animals are accidental hosts.
CHAPTER 7 Clinical Microbiology

Table 7–32 Zoonotic Infections and Causative Agents

<table>
<thead>
<tr>
<th>Zoonotic Infection</th>
<th>Causative Agent</th>
<th>Transmission</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plague</td>
<td>Yersinia pestis</td>
<td>Fleas</td>
</tr>
<tr>
<td>Lyme borreliosis</td>
<td>Borrelia burgdorferi</td>
<td>Ticks</td>
</tr>
<tr>
<td>Pasteureullosis</td>
<td>Pasteurella multocida</td>
<td>Cats and dogs–bites or scratches</td>
</tr>
<tr>
<td>Erysipeloid</td>
<td>Erysipelothrix rhusiopathiae</td>
<td>Contact with infected animals or animal products</td>
</tr>
<tr>
<td>Capnocytophaga canimorsus infection</td>
<td>Capnocytophaga canimorsus</td>
<td>Cat or dog bites</td>
</tr>
<tr>
<td>Cat scratch disease</td>
<td>Bartonella</td>
<td>Cat scratch</td>
</tr>
<tr>
<td>Bacillary rat-bite fever</td>
<td>Streptobacillus moniliformis</td>
<td>Rat bite; contaminated milk</td>
</tr>
<tr>
<td>Spinalilary rat-bite fever</td>
<td>Spirillum minus</td>
<td>Rat bite; contaminated milk</td>
</tr>
<tr>
<td>Anthrax</td>
<td>Bacillus anthracis</td>
<td>Inhalation; direct or indirect contact with animals or animal products</td>
</tr>
<tr>
<td>Tularemia</td>
<td>Francisella tularensis</td>
<td>Ticks, mosquitoes, rodents, and rabbits</td>
</tr>
<tr>
<td>Brucellosis</td>
<td>Brucella species</td>
<td>Contact with infected animals and animal products; contaminated milk</td>
</tr>
<tr>
<td>Leptospirosis</td>
<td>Leptospira interrogans</td>
<td>Contact with urine of animal reservoirs – rodents and domestic animals, contaminated soil or water</td>
</tr>
</tbody>
</table>

3. Epidemic typhus is caused by *R. prowazekii*. The infection is transmitted by lice and fleas. Flying squirrels and humans are the reservoirs.

4. Rickettsial pox, a spotted fever, is transmitted by mites and caused by *R. akari*. The reservoir is the common house mouse.

C. Other arthropod-borne infections include ehrlichiosis, scrub typhus, and Q fever.

1. Ehrlichiosis is transmitted ticks and natural hosts are dogs, deer, and other mammals. *Ehrlichia* species are major veterinary pathogens.

2. Scrub typhus is transmitted by chiggers and rats are the reservoirs. The etiologic agent is *Orientia tsutsugamushi*, a major veterinary pathogen.

3. Q fever is transmitted by ticks or inhalation of infected dust. It is caused by *Coxiella burnetti*.

4. Arthropod-borne infections caused by *Rickettsia*, *Ehrlichia*, *Orientia*, and *Coxiella* species are summarized in Table 7–33.

XXI. AGENTS OF BIOTERRORISM

A. Many highly infectious bacteria and viruses have been classified as potential agents of bioterrorism. A massive outbreak of infection in a population within a geographic area could result in high rates of morbidity and mortality, as well as contamination of food supplies, destruction of vegetation, and infections in livestock. Inhalation of aerosols or ingestion would be the most likely means of transmission.

1. Bioterroristic agents are classified in Categories A, B, or C according to their pathogenicity. Category A agents are the most infectious because of the potential

Table 7–33 Rickettsia, Ehrlichia, Orienta, and Coxella

<table>
<thead>
<tr>
<th>Arthropod-borne Infection</th>
<th>Infectious Agent</th>
<th>Arthropod Vector</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rocky Mountain Spotted Fever</td>
<td>Rickettsia rickettsi</td>
<td>Ticks</td>
</tr>
<tr>
<td>Endemic louse-borne typhus</td>
<td>Rickettsia typhi</td>
<td>Rat fleas</td>
</tr>
<tr>
<td>Epidemic louse-borne typhus</td>
<td>Rickettsia prowazekii</td>
<td>Lice and fleas</td>
</tr>
<tr>
<td>Ehrlichiosis</td>
<td>Ehrlichia species</td>
<td>Ticks</td>
</tr>
<tr>
<td>Scrub typhus</td>
<td>Orientia tsutsugamushi</td>
<td>Chiggers</td>
</tr>
<tr>
<td>Q fever</td>
<td>Coxiella burnetti</td>
<td>Ticks</td>
</tr>
</tbody>
</table>
Table 7–34 Category A Agents of Bioterrorism

<table>
<thead>
<tr>
<th>Bioterroristic Agent</th>
<th>Infection</th>
<th>Characteristic Clinical Manifestation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacillus anthracis</td>
<td>Anthrax – inhalational, gastrointestinal, and cutaneous</td>
<td>Black eschar</td>
</tr>
<tr>
<td>Francisella tularensis</td>
<td>Tularemia</td>
<td>Acute granulomatous disease; primary pleuropulmonary disease</td>
</tr>
<tr>
<td>Yersinia pestis</td>
<td>Plague – bubonic, pneumonic, and septicemic</td>
<td>Multi-system disease</td>
</tr>
<tr>
<td>Botulinum toxin</td>
<td>Botulism – wound, gastrointestinal, and infant botulism</td>
<td>Paralytic illness</td>
</tr>
<tr>
<td>Varicella major virus</td>
<td>Smallpox</td>
<td>Focal skin lesions progress to hemorrhagic smallpox</td>
</tr>
<tr>
<td>Hemorrhagic fever viruses</td>
<td>Hemorrhagic fevers</td>
<td>Severe multi-system syndrome with vascular hemorrhage</td>
</tr>
</tbody>
</table>

threat to humans and the ease with which these agents can be transmitted. These agents are listed in Table 7–34 with their respective clinical infections.

2. The Center for Disease Control (CDC) has a Laboratory Network Response (LNR) to decentralize testing capabilities and link state and local laboratories with advanced-capacity labs. LRN reference labs perform all confirmatory testing on biothreat agents.

3. Biosafety level laboratories are categorized in accordance with their capabilities to safely work with bioterrorist agents and minimize exposure to workers and the environment. Facilities are classified according to the safety equipment in the laboratory, as well as the training, procedures, and capability to perform adequate testing on the infectious agents. Laboratories are classified as BSL-1, BSL-2, BSL-3, and BSL-4. The most infectious agents are handled only in BSL-4 Laboratories, such as the CDC.

XXII. ANTIMICROBIAL SUSCEPTIBILITY TESTING

A. Background. The changing pattern of antimicrobial resistance of clinical isolates makes susceptibility testing of each isolate increasingly important. The in vitro results do not always give the complete picture. The antibiotic that should be used depends on other variables, such as host conditions, site of infection (e.g., CSF versus urine), route of administration, cost, and side effects.

B. Standardization. An important part of any susceptibility technique is use of a standard inoculum of bacteria. The most common procedure is to compare the turbidity of the inoculum with a McFarland turbidity standard. The 0.5 McFarland standard is usually used, and this is equivalent to 1.5 × 10^8 cfu/mL. The turbidity of a chemical precipitate in the standard correlates with the number of colony forming units of the bacterium per milliliter of inoculum. Regardless of the test used, each susceptibility method is highly standardized. The most commonly used tests are the Kirby-Bauer disk diffusion test and the minimum inhibitory concentration (MIC). Table 7–35 summarizes the antimicrobial susceptibility test procedures. Box 7–10 defines the areas of standardization that apply to both the Kirby-Bauer and MIC tests.

C. Interpretation of results. Results may be interpreted according to the following four categories:

1. Susceptible. The organism should respond to the usual doses of the drug.
2. Moderately susceptible. The isolate may be inhibited by concentrations of a drug that are achieved when the maximum parenteral doses are given.
3. Intermediate. The results are equivocal or indeterminate.
4. Resistant. The bacterium is not inhibited by achievable concentrations of drug.
Table 7–35 Summary of Antimicrobial Tests

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Definition</th>
<th>Specimen Tested</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minimum inhibitory concentration</td>
<td>Lowest concentration of antibiotic that inhibits visible growth</td>
<td>Bacterial isolate</td>
</tr>
<tr>
<td>Disk diffusion (Kirby-Bauer)</td>
<td>Measure diameter of growth inhibition around filter paper disk containing antibiotics</td>
<td>Bacterial isolate</td>
</tr>
<tr>
<td>Synergy</td>
<td>Activity of multiple antibiotics</td>
<td>Bacterial isolate</td>
</tr>
<tr>
<td>Serum bactericidal titer</td>
<td>Dilution of serum that kills 99.9% of the inoculum</td>
<td>Peak serum: trough serum, bacterial isolate</td>
</tr>
<tr>
<td>E-test</td>
<td>Elliptical zone of inhibition intersects with strip containing antibiotics in a concentration gradient to determine MIC</td>
<td>Bacterial isolate</td>
</tr>
<tr>
<td>β-lactamase test</td>
<td>Detection of bacterial β-lactamase enzyme; positive test indicates resistance to β-lactam antibiotics</td>
<td>Bacterial isolate</td>
</tr>
<tr>
<td>Antibiotic level</td>
<td>Concentration of antibiotic in serum</td>
<td>Peak serum; trough serum</td>
</tr>
</tbody>
</table>

D. The MIC is the lowest concentration of an antimicrobial agent that inhibits growth of the organism, as detected by lack of turbidity.

1. The antibiotic is added to broth in serial twofold dilutions (e.g., 0.5 mcg, 1.0 mcg, 2.0 mcg). A standardized inoculum of the test bacterium is added to each concentration of the antimicrobial agent. The MIC is the concentration of the first well that shows no growth or turbidity. Most routine MIC tests use commercially prepared broth microdilution trays with preselected antibiotics. MIC tube macrodilutions and microdilution trays are depicted in Web Color Images 7–87 and 7–88, respectively.

2. A sterility well monitors contamination. This well should have no growth. A growth well monitors organism growth and should have growth. If there is bacterial growth in the sterility well or the absence of growth in the growth well, the test is invalid and should not be read, but repeated.

3. Quality control testing with known bacterial strains (e.g., American Type Culture Collection (ATCC) strains) is performed weekly to ensure the validity of test results.

E. The Kirby-Bauer or disc diffusion (see Web Color Image 7–89) method measures the diameter of inhibition around an antibiotic-impregnated filter paper disk. A standardized bacterial suspension is plated to Mueller Hinton agar so as to achieve a confluent “lawn of growth.” Antibiotic disks are dropped on the agar surface. As soon as the disk comes in contact with the agar surface, water is absorbed into the filter paper, and the antibiotic diffuses into the surrounding medium. The concentration of the antibiotic decreases with increased distance from the disk. With overnight incubation at 37°C, a zone of inhibition surrounds the antibiotic disk in accordance with the bacterium’s susceptibility or resistance to the antimicrobial agent. If the test is properly performed, the edges of the zone of inhibition are clear and easy to measure. There are times when the zone is not obvious.

Box 7–10 Standardization of Susceptibility Testing

- Growth medium
- pH (7.2–7.4)
- Atmosphere (ambient air, no carbon dioxide)
- Temperature (35°C)
- Inoculum (McFarland 0.5 standard)
- Proper storage of antibiotic disks and MIC panels
- Quality control (use of stock cultures and regular control runs)
1. Swarming by *Proteus* may result in a thin film of growth beyond the outer margin. The zone of swarming should be ignored, and the outer margin should be measured.

2. Occasionally, colonies grow within the zone of inhibition. This represents either resistant mutants or a mixed culture. If the inoculum is a pure culture, the colonies represent a mutant, and the isolate is considered resistant.

3. The disks must be placed so that the zones of inhibition do not overlap. Overlapping zones may interfere with accurate interpretation of the results.

4. Quality control testing is performed weekly using stock cultures with known susceptibility results (e.g., ATCC strains).

F. The minimum bactericidal concentration (MBC) measures the lowest concentration of antibiotic that kills 99.9% of a bacterial isolate. It is used to demonstrate tolerance to an antibiotic. Tolerance is determined when the MBC endpoints are five or more twofold dilutions greater than the MIC.

G. Synergy describes the enhanced antibacterial activity achieved by using a combination of two drugs rather than either drug separately. This is helpful information in treating cases with combination drug therapy (e.g., *Escherichia coli*). A checkerboard MIC plate is used. Antibiotics are tested separately and together at various concentrations with the test bacterium to determine what concentration of each drug results in synergy.

H. The serum bactericidal titer tests the bacterial isolate from the patient with the patient’s own serum (containing the antibiotic). The lowest dilution of patient serum that kills a standard inoculum of the bacterium is called the serum bactericidal level. The Schlichter test is the most commonly used test protocol; however, it is not routinely performed.

I. E-test is similar to the disk diffusion test, but produces MIC results. Agar plates are inoculated in the same manner as those for disk diffusion tests. A plastic strip containing a gradient of the antimicrobial agent is placed on the plate. Following incubation, plates are examined for an elliptical zone of inhibition to determine the MIC value (see Web Color Image 7–90).

J. β-lactamase tests indicate that bacterial isolates produce β-lactamase, but cannot be used to predict the organism’s susceptibility or resistance to a particular drug. In general, β-lactamase-positive bacteria are usually resistant to the β-lactam antibiotics. Negative β-lactamase tests do not mean that an organism is susceptible to the β-lactam antibiotics. Direct β-lactamase tests are not appropriate for the Enterobacteriaceae and *Pseudomonas aeruginosa*.

K. Antibiotic levels in serum (e.g., gentamicin, vancomycin) are measured using immunoassays or other chemical methods to ensure that therapeutic levels of the antimicrobial agent are achieved in the serum. In addition, such monitoring ensures that toxic levels of the antibiotic are not present in the serum.

L. Antimicrobial agents include antiseptics, antibiotics, sterilants, and disinfectants. All have the capacity to kill or suppress the growth of microorganisms. Antibiotics are used to treat infections. These agents may be natural, semi-synthetic, or synthetic in accordance with how the agents are produced. Antibiotics may be grouped in accordance with the target site or mechanism of action. Antibiotic target sites include the following:

1. Interruption of structural integrity: interference with cell wall synthesis or cell membrane composition (e.g., penicillins, cephalosporins, carbapenems, vancomycin, and polymyxins)

2. Inhibition of protein synthesis (e.g., aminoglycosides, tetracyclines, macrolides, chloramphenicol)

3. Interference with nucleic acid metabolism (e.g., rifampin, metronidazole, nalidixic acid, fluorinated quinolones)
4. Inhibition of essential metabolites (e.g., sulfonamides, trimethoprim, Septra/Bactrim).

M. Some nonfastidious bacteria may require special procedures to detect clinically significant antibiotic resistance.

1. *Staphylococcus aureus*
   a. Methicillin-resistant (oxacillin-resistant) S. aureus (MRSA or ORSA) are resistant to the penicillinase-resistant penicillins, the drug class of choice for treating staphylococcal infections. The resistance is due to the presence of a penicillin-binding protein (PBP2a or PBP2') that is encoded by the mecA gene. Oxacillin screening with Mueller-Hinton agar supplemented with 4% NaCl and 6 mcg/mL oxacillin is used to detect MRSA. MRSA grows on the medium, whereas methicillin-susceptible strains are inhibited.
   b. Although uncommon, vancomycin-intermediate S. aureus (VISA) and vancomycin-resistant S. aureus (VRSA) have apparently acquired a plasmid containing the vanA vancomycin-resistant gene from vancomycin-resistant enterococci. The CLSI recommends use of broth microdilution (MIC) tests or vancomycin screening agar (BHI with 6 mcg/mL vancomycin) for detection of these bacteria.
   c. If a staphylococcal isolate is erythromycin-resistant but clindamycin-susceptible, the D-zone test must be performed for detection of inducible clindamycin resistance. The test is a disk diffusion test in which erythromycin and clindamycin disks are placed adjacent to each other on an inoculated agar plate. Following overnight incubation, a flattened clindamycin zone between the two disks indicates inducible clindamycin resistance due to the erm gene. The isolate must be reported as clindamycin-resistant. No flattening indicated the isolate is erythromycin-resistant only (due to msrA gene), and susceptible to clindamycin.

2. *Enterococcus* species
   a. Vancomycin-resistant enterococci (VRE) contain the vanA vancomycin-resistant gene that encodes for resistance. These are detected by use of the vancomycin agar screen agar plate (BHI with 6 mcg/mL vancomycin).
   b. High-level aminoglycoside resistance in enterococci is detected by use of broth, agar, or disk diffusion methods, testing for gentamycin and streptomycin resistance.

3. Resistance to later-generation penicillins, cephalosporins and aztreonam is due to extended spectrum β-lactamases (ESBLs) in *Klebsiella* species and *E. coli*. ESBL production is detected by disk diffusion screening or MIC breakpoints with the use of “indicator drugs,” followed by confirmatory testing. ESBL-producing strains should be reported as clinically resistant to all cephalosporins, penicillins, and aztreonam, regardless of in vitro susceptibility test results.

**XXIII. DISINFECTION AND STERILIZATION**

A. Disinfection is the elimination of a defined scope of microorganisms, including some spores.

  1. Disinfectants are chemical agents applied to inanimate objects.
  2. Antiseptics are applied to the skin to eliminate or reduce the numbers of bacteria present. These agents do not kill spores.
  3. Pasteurization & boiling achieve disinfection but not sterilization, as endospores are not destroyed.

B. Sterilization refers to the destruction of all life forms, including bacterial spores. There are chemical and physical means of sterilization.

  1. Physical means of sterilization include moist heat (autoclaves—heat under steam pressure), dry heat, filtration, and radiation.
  2. Chemical agents used for sterilization are known as chemosterilizers. These agents damage the bacterial cytoplasmic membranes, destroy cellular proteins, or damage bacterial RNA and DNA.
A Concise Review of Clinical Laboratory Science

**XXIV. MOLECULAR TESTING**

A. The increased use of molecular diagnostic techniques in the clinical laboratory is, in part, due to the increased sensitivity and specificity of these assays over routine culture techniques. The techniques allow for rapid detection of microorganisms and rapid answers for treatment options. Antibacterial resistance can be determined by the detection of resistance genes (e.g., the *vanA* gene in vancomycin-resistant enterococci). Three basic diagnostic techniques are as follows:

1. **Nucleic acid hybridization techniques**: detection of nucleic acid targets with labeled probes
2. **Amplicication techniques**: exponential increase of the target nucleic acid or the signal that binds to the target nucleic (e.g., PCR).
3. **Strain typing techniques**: used in epidemiologic studies to determine strain similarities in outbreaks

B. The simultaneous detection of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* from the same specimen is one example of molecular testing in use in the clinical microbiology laboratory. Refer to the chapter on molecular testing for a summary of diagnostic molecular procedures in the clinical laboratory.

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CHAPTER 8
Clinical Parasitology, Mycology, and Virology

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I. PARASITOLOGY

A. INTRODUCTION
1. Definition. Parasitism is a symbiotic relationship in which one animal, the host, is injured through the activities of the other animal, the parasite.


B. Phylum protozoa. The protozoa are eukaryotic (unicellular) and have organelles that function in nutrition or locomotion. The trophozoites (growing stage) feed by engulfing particles and move by flagella, cilia, or pseudopodia. The cyst stage is more resistant and typically is the form shed in stools and transmitted by the fecal-oral route.

1. Sarcodina. This subphylum of Protozoa includes Amoeba, which is a group of organisms that move by means of cytoplasmic protrusions called pseudopodia. Entamoeba histolytica is the most medically significant species in this group. E. histolytica is the agent of amebic dysentery, amebic liver abscess, and amebiasis. Other species, such as Entamoeba coli, Entamoeba hartmanni, Entamoeba gingivalis, Iodamoeba buetschlii, and Endolimax nana, are considered commensals. Identifying structures are summarized in Figure 8–1.

2. Sporozoa. This class is primarily tissue parasites with an involved life cycle that alternates between sexual and asexual generations. Plasmodium species are found as blood parasites and cause the disease malaria. Toxoplasma, Pneumocystis, and Sarcocystis species colonize the intestinal mucosa.

a. Plasmodium. The anopheline mosquitoes are definitive hosts of these malarial parasites. The female mosquito first must bite a human who is infected with malaria. Sexual reproduction occurs within the mosquito with the development of sporozoites. Sporozoites enter the salivary glands of the mosquito and are inoculated into the next person bitten. The sporozoites enter the liver and begin an asexual maturation stage. After several generations have developed, they invade the red blood cells (RBCs). The four species of Plasmodium are: P. vivax (benign tertian malaria), P. falciparum (malignant tertian or subtertian malaria), P. malariae (quartan malaria), and P. ovale (ovale malaria).
### Figure 8–1 Differentiation of Entamoeba histolytica and *E. coli.* (Reprinted from Johnson AG, Ziegler RJ, Lukasewycz OA, et al. *Microbiology and Immunology.* 2nd ed. Baltimore: Williams & Wilkins; 1993:188 with permission.)

1. Early forms of *P. vivax* in the RBC are trophozoites, which have a *signet-ring shape.* Later stages demonstrate fine red granules known as Schüffner’s dots. RBCs continue to enlarge, and the mature trophozoites begin division (at this point, they are referred to as schizonts). Schizonts mature into merozoites. The infected RBCs finally rupture to release merozoites to infect more RBCs.

2. *P. falciparum* gametocytes are elongated or *banana-shaped* compared to the ovoid morphology of the other *Plasmodium* species. The two stages observed are the young trophozoites and gametocytes. The young trophozoites are tiny ring forms. In later stages, Maurer’s dots (coarse red granules) are formed. Gametocytes have a distinct crescent shape.

3. *P. malariae* trophozoites demonstrate a *band form.* Schizonts mature into merozoites.

4. *P. ovale* demonstrates ovoid infected RBCs with early ring forms. Schüffner’s dots are present. In later stages, an average of 14 to 16 merozoites is observed.

b. *Toxoplasma.* The definitive host is the *cat.* Oocysts (containing two sporocysts) are passed in the feces. Transmission to humans occurs from consumption or handling of infected meat or from contact with cat feces in litter pans or soil. Serology is used for differential diagnosis.

c. *Isospora belli* is characterized by release of immature oocysts from the intestinal wall. Maturation of the oocyst occurs in the stool. The organism (30 μm) has an ellipsoid shape and contains two sporoblasts. Recovery is better with the zinc-sulfate concentration technique.

d. *Cryptosporidium parvum* causes chronic diarrhea in immunocompromised hosts. Diagnosis of cryptosporidiosis is based on observation of oocysts in the stool or biopsy material. Recovery of the *acid-fast* oocysts can be achieved by the sugar flotation procedure. Serologic testing allows for direct detection of the organism in fecal specimens.

3. *Mastigophora.* The organisms in this subphyla move by means of specialized *flagella.* (Figure 8–2). The number and position of the flagella vary according to species. The *Trypanosoma* are the blood flagellates. The *Leishmania* are the tissue flagellates. *Giardia* and *Dientamoeba fragilis* are intestinal flagellates, and *Trichomonas* can be...
Figure 8–2 Hemoflagellate forms. (Reprinted from Johnson AG, Ziegler RJ, Lukasewycz OA, et al. Microbiology and Immunology. 2nd ed. Baltimore: Williams & Wilkins; 1993:190 with permission.)

a. Trypanosoma. The three medically significant hemoflagellates are *T. brucei gambiense* (West African sleeping sickness), *T. brucei rhodesiense* (East African sleeping sickness), and *T. cruzi* (Chagas’ disease).

1. *T. brucei gambiense* is transmitted by *Glossina* (tsetse flies). The trypomastigotes reside in the blood stream, lymphatics, and cerebrospinal fluid (CSF). They are slender-bodied organisms with long, free flagella and an attached undulating membrane. A kinetoplast is posteriorly located, and the nucleus has a central karyosome.

2. *T. brucei rhodesiense* is a virulent form of trypanosomiasis transmitted by *Glossina*. Its structure is difficult to distinguish from *T. gambiense*.

3. *T. cruzi*. The reservoir of this organism is infected wild rodents. An intermediate host is the reduviid bug contained in rodent feces (not from bites). The trypanosome in blood smears has a “C” or “S” shape, a large kinetoplast, and a delicate undulating membrane. The amastigote stage is observed in cardiac muscle and other tissues.

b. Leishmania. The four major species (i.e., *L. tropica*, *L. braziliensis*, *L. mexicana*, *L. donovani*) are transmitted by the intermediate host the sandfly (*Phlebotomus* spp.). The amastigote form is observed in man, and the promastigote form resides in the gut of the sandfly.

1. *L. tropica* (Old World leishmaniasis, cutaneous leishmaniasis, Oriental sore) has a round amastigote form that can be observed in monocytic cells. It also has a rod-shaped kinetoplast with a single nucleus.

2. *L. mexicana* (New World leishmaniasis or cutaneous leishmaniasis) has the same structure as *L. tropica*.

3. *L. braziliensis* (mucocutaneous leishmaniasis or espundia) is disfiguring.

4. *L. donovani* (Kala-Azar or visceral leishmaniasis) demonstrates diffuse parasitization of the reticuloendothelial system. The amastigote resides within phagocytic cells and causes enlargement of the spleen, liver, and lymph nodes.

c. Giardia. *G. lamblia* (Figure 8–3) is the causative agent of giardiasis, which is the most common waterborne diarrheal disease in the United States.

1. The structure of the trophozoite is pear-shaped (9–16 μm); it contains four pairs of flagella; two prominent nuclei; an axostyle; and an oval, concave-sucking disk (“old man with glasses”). Motility is described as “falling leaf.”

2. The cyst (infective stage) is ovoid (9–12 μm) and contains two to four nuclei, as well as two longitudinal fibrils in the center.
d. **Trichomonas.** The three morphologically similar species are *T. tenax*, *T. hominis*, and *T. vaginalis*. Trichomonads appear only in the trophozoite form, which is the transmissible form.

1. *T. vaginalis* (Figure 8–3) is one of the main causes of sexually transmitted vaginitis. Differential diagnosis requires a saline wet mount of genitourinary tract specimens.

2. Trophozoites are pear-shaped with an undulating membrane. They demonstrate a jerky, swift motility.

e. **Chilomastix mesnili** is a commensal organism.

1. The trophozoite is pear-shaped with three anterior flagella and a nucleus.

2. The cyst is lemon-shaped with a large nucleus and a large karyosome.

4. **Ciliata. Balantidium coli** (Figure 8–3) has short, bristle-like cilia. *B. coli* is the largest protozoan that infects humans; it causes balantidial dysentery.

a. The trophozoite (60 μm) has two nuclei, and the cilia surrounds the organism.

b. The cyst (50 μm) has a macronuclei and a micronuclei. Cilia surround the membrane.

C. **Phylum Platyhelminthes.** The Platyhelminthes, or flatworms, are multicellular animals characterized by a flat, bilaterally symmetric body. Generally, the flat-worms are hermaphroditic. The two medically significant classes are the **Trematoda** and the **Cestoda**.

1. **Trematoda.** The flukes are organisms that possess attachment organs in the form of hooks or cup-shaped suckers. The medically significant, order is **Digenea.** The Digenea have life cycles that have at least one intermediate molluscan host.

a. **Fasciolopsis buski.** Reservoirs are pigs, dogs, and rabbits; snails are intermediate hosts. Transmission is ingestion of metacercariae that are encysted on freshwater vegetation, such as bamboo shoots or water chestnuts, which are consumed raw. The ellipsoid egg is approximately 130 μm, with a small operculum at the pointed end.

b. **Heterophyes heterophyes** and **Metagonimus yokogawai.** Transmission is through ingestion of raw or pickled freshwater fish. Metacercariae encyst under the scales or in the flesh of the fish. The egg is approximately 30 μm and possesses prominent opercular shoulders.
c. *Clonorchis sinensis*. The Chinese liver fluke is approximately 30 μm. Transmission occurs with the consumption of freshwater fish containing the encysted metacercariae. The adult worm harbors in the liver and sheds eggs into the feces. The eggs resemble *Heterophyes* but have a small comma-shaped process at the abopercular end.

d. *Fasciola hepatica*. The sheep liver fluke infection occurs following the consumption of aquatic vegetation (metacercariae encyst watercress). Eggs are observed in the feces and are difficult to distinguish from *Fasciolopsis*. The eggs are operculated and measure 130 μm.

e. The three medically significant schistosomes that parasitize man are *Schistosoma mansoni*, *S. japonicum*, and *S. haematobium*. The schistosomes are not hermaphroditic, they are diecious. The eggs are not operculated; the hatched miracidium swim in search of the appropriate snail host. The cercariae infect humans. Schistosomes directly penetrate the skin to invade the circulatory system. The final locations differ in each host. (1) *S. mansoni*. Eggs are isolated from the feces. They are elongated, ovoid, and possess a large lateral spine that is shaped like a thorn and projects from the side of the egg near one end. These eggs measure approximately 150 μm. (2) *S. japonicum*. Eggs are isolated from the feces. They are spherical in shape and possess a small lateral spine (sometimes this is absent or very difficult to view). These organisms measure approximately 65 μm. (3) *S. haematobium* is the cause of urinary schistosomiasis. Eggs are isolated in urine or in a biopsy of the bladder. The eggs have a terminal spine and measure approximately 150 μm.

f. *Paragonimus westermani*. The lung fluke is transmitted by the ingestion of raw or improperly cooked crayfish or freshwater crabs encysted with the metacercarial stage. The eggs are discharged into the bronchi or bronchioles; they can be expectorated in sputum or, if swallowed, appear in the feces. The ovoid eggs have raised opercular shoulders and measure approximately 100 μm.

2. *Cestoda*. The tapeworms have elongated segmented (proglottid) bodies with a scolex (specialized attachment organ). They inhabit and gain nourishment from the small intestine. All cestodes of man have four muscular, cup-shaped suckers on the scolex, and a rostellum that can be armed with hooks. Transmission occurs when infective larvae are accidentally ingested with food.

a. *Diphyllobothrium latum* selectively absorbs vitamin B12 from the host’s intestinal tract. The primary intermediate host is the ciliated coracidium larva (which has ingested the *D. latum* eggs). The larvae are ingested by* copopods* (*D. latum* develops and forms larvae). These infected cope-pods are then ingested by fish, and *D. latum* grows within the flesh of the fish to the plerocercoid larval stage. Only if the infected fish is ingested by man will the larva pass into the intestine and develop into adulthood. (1) The adult worm scolex is elongated, spoon shaped, and has two longitudinal grooves. The uterus, within the proglottid, has a unique morphology and forms a rosette. (2) The eggs are ovoid with an operculum and a thick, smooth shell. They are approximately 70 μm.

b. *Taenia solium*. The pork tapeworm is transmitted by ingesting cured or undercooked pork. Embryonated eggs are ingested by the intermediate host (the pig) and develop into the infective larval stage or *cysticerci* in the muscles. Humans ingest the cysticerci; the larvae develop, and the scolex attaches to the intestinal wall and begins to develop proglottids (cysticercoisis). (1) Structure. The adult worm scolex has four suckers and a double crown of hooks (armed rostellum). Differential diagnosis is by observation of 7-13 branches of the central uterine stem in a gravid segment. (2) The egg contains the oncosphere. A thick coat, called the embryophore, is a dark color, and is radially striated. Observation of *Taenia* eggs cannot differentiate *T. solium* from *T. saginata*.
e. *Taenia saginata*. The beef tapeworm is transmitted by the ingestion of raw or insufficiently cooked beef. Embryonated eggs are ingested by cattle and develop in the flesh into the infective cysticercus stage. (*T. saginata* does not cause cystercosis of humans.)

1. The adult worm scolex has four suckers with a small rostellum that has no hooks (unarmed rostellum). The gravid segment is longer in *T. saginata*, and there are 15 to 20 branches off of the central uterine stem.

2. Morphology of the eggs of *T. saginata* is similar to that of *T. solium*.

d. *Echinococcus granulosus*. The minute tapeworm lives as an adult in the intestines of the Canidae. Intermediate hosts are typically herbivores (e.g., sheep, goats). The human is an accidental intermediate host. Humans can ingest the eggs, which hatch and develop into oncospheres. The oncospheres penetrate the intestinal mucosa, enter the circulation, and progress to the liver. A hydatid cyst begins to develop, with buds forming on the cyst. The cysts contain protoscolices, which appear as hydatid sand when the cyst begins to break down.

1. The adult worm has a scolex (with six hooklets) and three proglottids.

2. Structure. Eggs cannot be differentiated from *Taenia* eggs.

e. *Hymenolepis nana*. The dwarf tapeworm is transmitted to humans by ingestion of the egg, which develops into the adult worm in the intestines of the human. The reservoir is the house mouse.

1. The adult worm has a scolex with four suckers and a rostellum with a circle of hooks.

2. The eggs are ovoid and have a thin, smooth outer shell with two polar thick-enings or knobs that have fine filaments (4–8) that are difficult to observe. The eggs are approximately 40 μm.

f. *Hymenolepis diminuta* transmission is through ingestion of infected insects from flour contaminated by the droppings of infected rats. Larvae grow into adults in the intestine of humans.

1. Adult worms have a scolex with four suckers and an “unarmed” rostellum.

2. Eggs are ovoid with a thick shell that also has two polar knobs, but no filaments.

D. Phylum Aschelminthes. The medically significant class is Nematoda, the roundworms. They possess a stiff cuticle, and the sexes are separate. The roundworms can inhabit the intestines, the blood, or the tissues. The filariae are thread-like worms that inhabit the human lymphatic system, as well as subcutaneous and deep connective tissues. All of the filariae require an arthropod intermediate host for transmission of infection. Some species demonstrate periodicity. That is, they appear in the blood at specific times of the day.

1. *Ascaris lumbricoides*. The life cycle of *Ascaris* is complex. After the fully embryonated eggs are ingested, they hatch in the intestines and begin to migrate (up to 20 days) through the blood and lymphatic system. They finally reach the esophagus and once again enter the intestines, where the mature worm then begins to lay eggs.

a. Adult worm

1. The adult female worm is 30 cm in length and is as thick as a lead pencil.

2. The adult male worm is 25 cm in length and is slender with an “incurved tail.”

b. The eggs are broadly oval with a rough outer coating, an albuminoid coat. They are referred to as fertilized corticated eggs. Decorticated eggs have a thick, smooth shell. Infertile eggs are narrow and have a thin shell (corticated or decorticated).

2. *Enterobius vermicularis*. The pinworm is the most common helminth parasite in modern countries. Transmission is by ingestion of the eggs, which pass to the intestine and begin development. The females migrate down the intestinal tract to pass out of the anus and deposit their eggs. The cellophane tape preparation is useful for diagnosis. Typically, the eggs are not observed in the feces.

a. Structure

1. The adult female worm can reach 13 mm in length. It has a long, thin, sharply pointed tail.

2. The adult male worm reaches 5 mm in length.
b. The eggs are 50 μm and have a translucent shell that is flattened on one side (football appearance).

3. Trichuris trichiura. The whipworm is acquired through ingestion of embryonated eggs. It can cause rectal prolapse and nutritional deficiencies in children.
   a. The female worm measures 3 to 5 cm and is typically longer than the male.
   b. The eggs are 50 μm and have bipolar plugs.

4. Ancylostoma duodenale. The Old World hookworm eggs are deposited into moist sandy soil, and within 24 to 48 hours rhabditiform larvae develop and hatch. Further development transforms the larvae into the infective filariform larvae. The filariform penetrate the skin of humans. Humans may develop ground itch at the site of penetration. Hookworm infestation is associated with iron deficiency anemia.
   a. Adult worm
      (1) The female worm is grayish white with a buccal cavity that has four teeth.
      (2) The male worm is smaller than the female worm and has a prominent copulatory bursa posteriorly.
   b. The eggs of A. duodenale and Necator americanus cannot be differentiated. The eggs are approximately 50 μm with a thin, smooth colorless shell. Clearly visible are the two-cell, four-cell, or occasionally eight-cell stage of embryonic cleavage.

5. Necator americanus is the New World hookworm.
   a. Adult worm
      (1) The female worm is 1 cm in length. The buccal cavity of Necator is armed with a pair of cutting plates.
      (2) The male worm is smaller than the female worm.
   b. Eggs (see Section ID 4 b)
   c. Rarely, hookworm eggs can hatch and release the rhabditiform larvae. The larvae can be differentiated from Strongyloides larvae by observing the buccal cavities. The hookworm buccal cavity has a long capsule between the oral opening and the esophagus. The Strongyloides buccal capsule is comparatively short.

6. Strongyloides stercoralis. The threadworm is less prevalent than the hookworm and can exist as a free-living nematode. Transmission and life cycle are similar to the hookworm.
   a. Adult worm
      (1) The female worm is longer than 2 mm and is parthenogenic (capable of unisexual reproduction, no fertilization required).
      (2) The male worm is smaller, and it may be eliminated from the body early in infection.
   b. Structure. Eggs of the threadworm cannot be distinguished from the hookworm eggs. The eggs hatch in the intestine to form the rhabditiform larvae and are usually passed in the feces. Occasionally, a few of these larvae transform into the filariform larvae, which penetrate the mucosa wall, enter the bloodstream, and begin the migratory cycle again. This is termed aut reinfection.

7. Trichostrongylus spp. This worm is very similar to the hookworm. The life cycle differs in that Trichostrongylus is transmitted by ingestion of contaminated food. The adult worms are much smaller than the hookworms. The eggs are much larger than the hookworm eggs (80 μm).

8. Trichinella spiralis. Trichinosis and trichinellosis are acquired by ingesting uncooked pork or bear meat that contains the encysted larvae. The larvae encyst and penetrate into the intestinal mucosa of the host and develop into adult worms. The male and female worms mate, and more larvae are produced (intestinal phase). The larvae enter the bloodstream and disseminate to the striated muscle, where they encyst. Diagnosis is based on observation of the encysted larvae in biopsied muscle or postinfection serologic testing.

9. Dracunculus medinensis. The guinea worm is a parasite of dogs and other carnivores in North America.
   a. Infection. The larvae are ingested by the intermediate host—the copepod (crustacean)—and develop into the infective form. The infective larvae are
ingested in drinking water and migrate in the host to the subcutaneous tissues. The larvae develop into the adult worm. The female worm migrates to the skin surface and forms a \textit{papule} to discharge large numbers of larvae.

\textbf{b. Structure}

(1) The adult female worm is approximately 800 mm in length and can be removed from the papule manually or excised surgically.

(2) The adult male worm is approximately 40 mm in length.

10. \textit{Ancylostoma braziliense} or \textit{A. caninum} are the \textit{dog hookworms} that occasionally infect humans and cause \textit{cutaneous larva migrans} or creeping eruptions. The larvae penetrate the skin and are unable to complete their migratory cycle; therefore, they begin to migrate through the subcutaneous tissues.

11. \textit{Wuchereria bancrofti}. \textit{Bancroftian filariasis}, \textit{elephantiasis}, or \textit{Bancrofti’s filariasis} are associated diseases.

a. \textit{Infection}. The infective larvae enter the human host through the proboscis of the \textit{Culex} or \textit{Anopheles} mosquito. They migrate to the lymphatics (lymph nodes), where mating occurs and microfilariae are produced (can be observed in a blood smear).

b. \textit{Structure}. The microfilariae are sheathed. The anterior is rounded, and the posterior tapers to a point. There are no nuclei in the tail.

12. \textit{Brugia malayi}. \textit{Malayan filariasis} or \textit{elephantiasis} is transmitted by the \textit{Mansonia} or \textit{Anopheles} mosquito.

a. \textit{Infection}. Migration occurs through the lymphatics. Microfilariae can be observed in the blood smear.

b. \textit{Structure}. The microfilariae are sheathed. There are two nuclei present in the tip of the tail.

13. \textit{Loa loa}. The \textit{African eyeworm} is transmitted by the \textit{Chrysops} (fly) and migrates through the subcutaneous tissues (especially across the conjunctiva of the eye). Calabar swellings also occur. The microfilariae can be observed in the blood. They are sheathed and have a continuous row of posterior nuclei.

14. \textit{Onchocerca volvulus}. \textit{Onchocerca} is transmitted by the \textit{Simulium} fly and migrates through the subcutaneous tissues. They usually settle in groups of two and become encapsulated to form nodules. These nodules can occur anywhere, but are typically observed on the patient’s scalp. The microfilariae can be observed in “skin snips” taken from the nodules. The microfilariae are unsheathed, with no terminal nuclei, and typically have straight tails.

15. \textit{Mansonella}

a. \textit{M. ozzardi} is typically a nonpathogenic filaria. The microfilariae have no sheaths and no nuclei in their tails.

b. \textit{M. streptocerca} (formerly \textit{Dipetalonema streptocerca}) is pathogenic and transmitted by \textit{Culicoides}. The microfilariae are observed only in tissue scrapings. They have no sheaths and have single nuclei in their tails.

c. \textit{M. perstans} (formerly \textit{D. perstans}) is nonpathogenic. The microfilariae have no sheaths and have paired nuclei in their tails.

\textbf{E. Specimen collection, transport, and processing}

1. Collection and transport for \textit{fecal examination} should include the following precautions.

a. The specimen should be collected in a clean container and must not be contaminated with urine.

b. If not examined within 1 hour, part of the specimen should be preserved in polyvinyl alcohol fixative (PVA) or in 10\% formalin.

(1) PVA preserves cysts and trophozoites for permanent staining

(2) Formalin preserves eggs, cysts, and larvae for wet-mount examination and concentration.

c. Three specimens every other day is the optimal number for recovery of parasites.

d. Saline purges are satisfactory to obtain specimens; castor and mineral oils make examination for protozoa impossible.
e. Specimens containing radiographic contrast media (e.g., barium salts) are inappropriate.

2. Processing of specimens. The processing should include a macroscopic examination, a microscopic examination, and a concentration procedure.
   a. The process of macroscopic examination involves the following:
      (1) Noting the consistency of the specimen (i.e., soft stools suggest the presence of trophozoites or protozoa; formed stools suggest the presence of cysts)
      (2) Examining the surface for tapeworm proglottids or adult pinworms
      (3) Examining for blood and mucus
   b. The microscopic examination should include a direct saline and/or iodine mount. A calibrated ocular micrometer should be used routinely. Size differences of various internal structures and of whole organisms are important in differential diagnosis.
   c. A fecal concentration technique increases the possibility of detecting parasites when few are present in feces; this technique should be a routine part of the examination process.
      (1) The Ritchie formalin-ether method is the most commonly used method for concentrating eggs and cysts.
      (2) The zinc sulfate flotation method is not appropriate for operculated eggs, schistosomes, or infertile ascaris eggs. This method is recommended for recovery of oocysts.

3. The trichrome technique is a rapid procedure that gives good results for routine identification of intestinal protozoa in fresh fecal specimens. The specimen must be fixed with either PVA or Schaudinn’s solution.
   a. The cytoplasm of trophozoites and cysts appears light blue-green or light pink.
   b. Karyosomes stain ruby red.
   c. Degenerated organisms and the background material stain pale green.

II. MYCOLOGY

A. INTRODUCTION

1. Definition. Mycology is the study of the eukaryotic fungi, including yeasts, molds, and mushrooms.

2. Characteristics of fungi include the following:
   a. The eukaryotic fungi are non-motile and have definite cell walls. They lack chlorophyll and absorb nutrients.
   b. Hyphae are branching filaments of molds and mushrooms. The mycelium is the mass of hyphae constructing the thallus, the vegetative body of a fungus.
      (1) Hyphae may be dematiaceous (dark) or hyaline (colorless).
      (2) Hyphae can be septate (with crosswalls) or aseptate. Aseptate fungi are often referred to as coenocytic.
      (3) Vegetative, or food-absorbing, hyphae are either submerged or on top of the agar substrate. Aerial hyphae extend above the agar surface and may support reproductive structures, commonly called conidia.
   c. Pseudohyphae are a series of elongated blastoconidia made by some yeast. These remain attached to each other and form a hyphal-like structure. Points of attachment and septations are constricted, while those of true hyphae are not constricted. (characteristic of Candida albicans).
   d. Spores or conidia may be formed by either an asexual or a sexual process.
      (1) Asexual spores
         a. Conidia are asexual spores formed from a conidiogenous cell or at the end of conidiophores. The conidial types are used to identify the Fungi Imperfecti, or phylum Deuteromycota. These fungi have no known sexual stage.
         b. Eastic conidiogenesis results in the production of blastoconidia that bud from a mother cell. They can elongate to form pseudohyphae. These
conidia are produced by yeasts and some fungi. Poroconidia, phialoconidia, and annelloconidia are all produced by blasto conidiogenesis.

(e) In the process of thallic conidiogenesis, the daughter conidium develops after the formation of a septum near the end of a parent cell. Macroconidia and microconidia, characteristic of the dermato phytic fungi, are produced by thallic conidiogenesis.

(d) Arthric conidiogenesis results in the formation of arthroconidia, which fragment from the hyphal strand at the septation points. This is a type of thallic conidiogenesis.

(a) Chlamydoconidia, seen in molds, are thick-walled survival conidia that can occur either at a terminal site (end), an intercalary site (within the hyphae), or a sessile site (on the sides). These germinate and produce conidia under favorable environmental conditions.

(f) Sporangiospores are formed by internal cleavage of the contents of a sporangium, a sac that is supported by a sporangium. This type of free cell formation is characteristic of the Zygomygetes.

(2) Sexual spores are produced by the Perfect Fungi.
(a) Zygospores are formed by fusion of two compatible hyphal arms and are surrounded by a thick walled, protective zygosporangium. These sexual spores are produced by fungi in the phylum Zygomycota.
(b) Ascospores are sexual spores produced by fungi in the phylum Ascomycota. Eight ascospores are inside an ascus that may be surrounded by a protective ascocarp.
(c) Basidiospores are sexual spores that protrude out of a mother cell called a basidium. The basidiospores and basidium may be protected by an outer basidiocarp, as in the mushrooms, members of the phylum Basidiomycota.

B. Specimen collection and transport
1. Collection of specimens for mycologic cultures must include:
   a. Sterile technique
   b. Adequate amount
   c. Sample from the area most likely affected
2. Transport
   a. Blood and bone marrow are collected into a brain heart infusion (BHI) broth for transportation to the laboratory. The use of Dupont Isolator tubes for transport and processing enhances fungal recovery from blood and bone marrow specimens.
   b. CSF is aseptically collected and transported immediately to the laboratory.
   c. Hair, nails, and skin are initially cleaned with 70% alcohol to remove surface contaminants. Scrapings and plucked hairs are placed in a sterile Petri plate for transportation.
   d. Respiratory tract specimens (e.g., sputum) should be collected in the morning and put into a sterile container for transport.
   e. Tissues and biopsy specimens are aseptically collected and kept moist with sterile saline for transport.
   f. Vaginal and cervical specimens are typically collected on sterile swabs, then placed into transport media or broth.
   g. Scrapings from wounds and lesions may be placed into sterile saline for transport. Aspiration specimens can be collected from deep cysts or abscesses by needle and syringe.
   h. Urine specimens are collected in the morning in sterile container and sent immediately to the laboratory. Clean catch or catheterized specimens are recommended.

C. Media and tests
1. Sabouraud dextrose agar (SDA) inhibits the growth of many bacteria because its pH is 5.6. However, this medium allows fungal contaminants and pathogenic fungi to grow. Histoplasma capsulatum and some strains of Nocardia asteroides fail to grow on this medium.
2. SDA with cycloheximide and chloramphenicol is reserved for skin, hair, and nail specimens.
3. BHI agar (BHIA) is very nutritious and supports the growth of bacteria and fungi, including *Histoplasma* and *Nocardia*.
   a. BHIA with blood is recommended for converting dimorphic fungi from the mold to the tissue (yeast) phase.
   b. BHIA with blood, cycloheximide, and chloramphenicol is very nutritious but inhibits the growth of *Nocardia*. (NOTE: Chloramphenicol inhibits the yeast phase of dimorphic fungi.) It supports the growth of dermatophytes, isolated from skin, hair and nail specimens.
4. Corn meal Tween 80 agar (CMT 80) is used for the demonstration of blastoconidia, pseudohyphae, arthroconidia, and chlamydospores in the identification of *Candida* species and other yeasts.
5. Potato dextrose agar (PDA) is a medium used to enhance sporulation and pigmentation. It is used as a subculture medium rather than a primary plating medium.
6. Inhibitory mold agar is used primarily to recover pathogenic fungi exclusive of dermatophytes.
7. Mycosel agar is used to recover dermatophytes.
8. Dermatophyte test medium (DTM) is a screening medium used for the isolation of dermatophytes.
9. Birdseed agar (Caffeic Acid Agar) is used for growth of *Cryptococcus neoformans*.
10. Trichophyton Test Agars are used for speciation of the *Trichophyton* species.
11. Germ tube media (rabbit, fetal calf, or human serum) is used to demonstrate germ tube production by the yeast *Candida albicans*.
12. Urea agar slant is used to demonstrate urease production. A positive test is development of a pinkish purple within 48 hours after inoculation.
   a. *Trichosporon*, *Rhodotorula*, and *Cryptococcus* are urease-positive.
   b. *Geotrichum*, *Saccharomyces*, and most *Candida* are urease-negative.

D. Microscopy of clinical specimens
1. Saline wet mounts allow the observation of budding yeast, hyphae, conidia, and hyphal filaments. These mounts are primarily used for the observation of vaginal specimens.
2. Lactophenol cotton blue stain (LPCB) can be used for direct smears or stains of fungal isolates. Tease mounts for permanent smears and scotch tape preps can be made using this stain.
   a. Phenol kills organisms.
   b. Lactic acid preserves the fungal structures.
   c. Cotton blue stains the chitin in the fungal cell walls.
3. Potassium hydroxide (KOH) is ideal for observing skin, hair, or nails. Ten percent potassium hydroxide dissolves the keratin and enhances visibility of the fungi.
4. Gram’s stain. The fungi appear gram-positive or blue. This stain is used primarily to observe yeast and pseudohyphae present in clinical specimens.
5. Acid-fast stain. The modified Kinyoun acid-fast stain is recommended for staining the partially acid-fast *Nocardia*. The fluorescent auramine-rhodamine acid-fast stain may also be used.
6. India ink. This background stain is recommended for observing encapsulated yeast, especially *Cryptococcus neoformans*.
7. Periodic acid-Schiff (PAS) stains fungi (except *Actinomyces*) magenta against a light pink or green background.
8. Calcofluor white stain is observed using a fluorescence microscope. With this sensitive stain, fungal elements fluoresce against a dark background. Ten percent KOH may be added to dissolve keratin in the specimen.

E. Opportunistic fungi are exogenous saprophytes capable of causing infections in immunocompromised individuals. These fungi are routinely encountered in routine fungal cultures and must be differentiated from pathogenic fungi. This group of fungi includes the asperate *Zygomycetes*, as well as many septate *Deuteromycetes* and *Ascomycetes*. Characteristic
Asexual conidial forms are primarily used to identify and differentiate these fungi. Production of sexual spores may be used to differentiate the Zygomyces.

1. Hyaline (light) opportunists–septate fungi
   a. *Acremonium* species microscopically demonstrate tapering conidiophores that support closely packed balls of sickle- or elliptical-shaped conidia.
   b. *Aspergillus* species have rough or smooth unbranched conidiophores with a foot cell at the base and a vesicle (head) at the tip. Rows of flask-shaped phialides in single or double rows support chains of rough or smooth phialoconidia.
      (1) *A. fumigatus* is the most common opportunistic pathogen in the genus. It grows at 45°C, and the phialides cover the upper two thirds of the vesicle. Colonies are dark greenish to gray.
      (2) *A. niger* has a black radiate head with phialides and chains of phialoconidia covering the entire vesicle. Colonies are dark black.
      (3) *A. flavus* has spiny (rough) conidiophores and phialides cover the entire vesicle. Colonies are usually a yellow-green color.
      (4) *A. terreus* produces cinnamon-brown colonies.
   c. *Fusarium* species have single or branching conidiophores that terminate in tapered phialides. Macrophialoconidia are banana or cylindrical-shaped with two to five cells.
   d. *Gliocladium* species produce terminal masses of phialoconidia supported by flask-shaped phialides. Terminal masses of phialoconidia are held together in a large ball by a gelatinous matrix. Mature colonies are a characteristic “green lawn” of growth.
   e. *Paecilomyces* species have single, short, or penicillus-type phialides with very delicate chains of smooth or rough, oval conidia. This fungus resembles *Penicillium*.
   f. *Penicillium* species colonies are characteristically blue-green with a white peripheral. Multiple, flask-shaped phialides support chains of round phialoconidia. Microscopically, the fungus has a “brush-like” appearance.
      (1) *P. marneffei* is a dimorphic fungus that produces yeast-like colonies at 37°C. In the mycelial form (25–30°C), chains of lemon-shaped phialoconidia are supported by wide phialides on broad metulae (conidiophores).
   g. *Scopulariopsis* species have echinulate (spiny) lemon-shaped annelloconidia in chains, supported on flask-shaped annellides. Unbranched annellides support the annellides.
   h. *Sepedonium* species have simple or branched conidiophores supporting single or clustered thick-walled, smooth or rough macroconidia. This hyaline fungus resembles the yeast phase of the dimorphic pathogen *Histoplasma capsulatum*.

2. Dematiaceous (dark) opportunists–septate fungi
   a. *Alternaria* species colonies are dark brown to black, and the hyphae and conidia are dematiaceous. Chained poroconidia with horizontal and vertical septa have club-shaped bases with tapered apices.
   b. *Bipolaris* species have dark, septate hyphae with cylindrical, four- to five-celled poroconidia. “Bent-knee” shaped conidiophores support the poroconidia, with truncate hila at the points of attachment. Bipolar germ tube formation is produced by these fungi.
   c. *Cladosporium* species produce short chains of blastoconidia from repeatedly forking shield cells. There is a scar at the point of attachment to the shield cells.
   d. *Curvularia* species have large four- to five-celled poroconidia with an over-enlarged central cell and bent-knee conidiophores.
   e. *Exserohilum* species have “bent-knee” conidiophores and protruding truncate hila at the ends of dark cylindrical poroconidia with 6 to 14 cells.
   f. *Epicoccum* species have thick clusters of sporodochia (short conidiophores) that support terminal dark, round conidia with horizontal and vertical septa.
   g. *Nigrospora* species have dark hyphae and short fat conidiophores supporting single, oval, smooth-walled, black conidia.
3. Zygomycetes—aseptate opportunists with large, ribbon-like hyphae
   a. *Absidia* species have stalk-like, branching sporangiophores with a columella (base) supporting pear-shaped sporangia (a sac). Asexual sporangiospores are produced within the sporangia by free-cell formation. These aseptate Zygomycetes have rhizoids, or root-like structures, between the interconnecting runners (stolons) connecting the sporangiophores.
   b. *Mucor* species have single or branching sporangiophores supporting round, spore-filled sporangia. There are no rhizoids or stolons.
   c. *Rhizopus* species produce black sporangia on unbranched sporangiophores opposite rhizoids. Stolons connect the groups of rhizoids.
   d. *Saksenaea* species have large, flask-shaped sporangia filled with elongated sporangiospores. Rhizoids are opposite the sporangiophores.
   e. *Cunninghamella* species have large, flask-shaped sporangia filled with elongated sporangiospores. Rhizoids are opposite the sporangiophores.
   f. *Syncephalastrum* species produce terminal, round vesicles. Denticles protrude from the vesicles and support one-celled round sporangiola.

4. Single-celled yeasts are normal flora of the skin, mucous membranes, and gastrointestinal tract and can be opportunistic pathogens.
   a. *Candida albicans* is predominantly the causative agent of these opportunistic infections.
      (1) *C. albicans* form chlamydospores, blastoconidia, and pseudohyphae on CMT 80 agar. Germ tubes are formed following incubation in germ tube media.
      (2) *C. albicans* assimilates the carbohydrate sucrose.
   b. *Torulopsis glabrata* is normal vaginal flora and may be associated with vaginal and urinary tract infections.
   c. Emerging opportunistic pathogens are *C. tropicalis*, *C. parapsilosis*, and *C. krusei*.

F. Opportunistic mycoses include infections ranging from mild and self-limiting to serious disseminated infections in severely immunocompromised individuals. These mycoses are caused by exogenous, saprophytic fungi, and they are increasing as the number of immunocompromised patients increases.

1. *Candidiasis* is an acute-to-chronic fungal infection that can involve the mouth, vagina, skin, nails, bronchi, lungs, alimentary tract, bloodstream, or urinary tract. Typically, the causative agent is *Candida albicans*, but other species of *Candida* are emerging as opportunistic pathogens. *Candida albicans* is part of the normal flora of the skin, mucous membranes, and gastrointestinal tract.
   a. Oral thrush is a yeast infection that forms white curd-like patches on the oral muco-cutaneous membranes.
   b. Vulvovaginitis or vaginal thrush manifests as a thick yellow-white discharge. Diabetes, antibiotic therapy, oral contraceptives, and pregnancy predispose the patient to this condition.
   c. Candidemias, primarily caused by *C. parapsilosis*, occur in patients who have indwelling catheters.
   d. Other opportunistic yeast infections in debilitated patients may be caused by *Geotrichum candidum* and *Torulopsis glabrata*. Infections range from oral and urinary tract infections to disseminated infections.

2. *Malassezia furfur* can be an opportunist, causing septicemia in patients who receive intravenous lipid infusions. The fungus grows well in the sebaceous glands. It is also the causative agent of the superficial mycoses pityriasis versicolor.
   a. *M. furfur* requires a medium overlayed with a lipid, such as olive oil.
   b. Microscopically, this septate fungus appears as thick, round oval cells in clusters with hyphae (spaghetti and meatballs).

3. Cryptococcosis infections primarily involve the lungs and meninges and often occurs in AIDS patients. The yeast *Cryptococcus neoformans* is the causative agent of cutaneous and disseminated disease. Inhalation of fungal spores is usually the means of transmission.
a. *C. neoformans* has a capsule that is demonstrated with the India ink preparation. It is urease-positive.

b. *C. neoformans* is associated with pigeon and bird feces. It may also be associated with fruits, milk, or plants.

c. Capsular material in the CSF can be detected with an antigen latex agglutination test.

4. Aspergilloses are a variety of infections and allergic diseases primarily caused by *Aspergillus fumigatus*, *A. flavus* and *A. niger* may also be causative agents of these infections. The *Aspergillus* species are ubiquitous fungi with airborne spores.

a. Allergic aspergillosis involves inhalation of the conidia, colonization of the mucous plugs in the lung, and subsequent asthma attacks. A high titer of IgE antibody to *Aspergillus* is present.

b. Otorrhea (outer ear infections) is a characteristic form of aspergillosis. However, there is no tissue invasion.

c. Fungus ball, a localized abscess that forms in the lung, is another form of aspergillosis. However, there is no tissue invasion.

d. Invasive aspergillosis occurs in neutropenic patients. Initially, the patient has sinusitis, followed by dissemination throughout the body (e.g., brain, bones) occurs.

5. Penicilliosis is caused by the penicillus-producing fungi, *Penicillium*, *Scopulariopsis*, and *Paeceilomyces*. Infections may include otomycosis (outer ear), keratomycosis (cornea of the eye), infections of the nails. Dissemination occurs in debilitated patients with transmission via inhalation of spores and spread of an initial pulmonary disease. The dimorphic fungus, *P. marneffei*, causes a disseminated form of penicilliosis with flu-like symptoms, followed by enlarged lymph node, spleen, and liver. Transmission may be via inhalation or ingestion of the fungal spores.

6. Zygomycoses, also known as phycomyces or mucormycoses, are acute infections caused by the *asепtate fungi* in the phylum Zygomycota: *Rhizopus*, *Absidia*, and *Mucor*. Predisposed patients have uncontrolled diabetes, malnutrition, or are on steroid therapy. Spores infect the nasal sinuses or orbital (eye) area and the infection disseminates rapidly. Without treatment, death ensures within two to ten days after the infection. Prompt treatment with amphotericin B is usually successful if initiated at the beginning of the infection.

7. Other opportunistic fungal mycoses may include keratomycosis (corneal infections) and otomycosis (outer ear infections). These infections are usually the result of trauma. Acute or chronic sinusitis may also be caused by opportunistic fungi.

G. Systemic mycoses affect internal organs and disseminate to multiple organs in the body. Primary or secondary cutaneous infections are often characteristic of these mycoses. Causative agents are typically the slow-growing, dimorphic fungal pathogens. These fungi (grow as molds at 25°C and as yeast at 35°C).

1. Histoplasmosis, or *Darling’s disease*, is a granulomatous fungal infection caused by *Histoplasma capsulatum*. The progressive disease is transmitted via inhalation or direct inoculation into the skin, and is not contagious or transmissible via person-to-person.

a. The yeast or tissue phase (37°C) is small, single-budding yeast cells.

b. The mold (25°C–30°C) demonstrates is a large tuberculate macroconidia and small rough or smooth microconidia along the sides of the hyphae. This is the diagnostic stage. The fungus resides in soil enriched with bat or bird guano (inhalation transmission).

c. The major endemic region is the Ohio, and Mississippi River areas, and along the Appalachian Mountains.

2. North American blastomycosis, or *Gilchrist’s disease*, is caused by *Blastomyces dermatitidis*. Transmission is inhalation of the conidia. The primary respiratory disease gradually disseminates hematogenously to other organs.

a. The diagnostic tissue (37°C) phase demonstrates large yeast with a double refractile wall and a broad-based bud.

b. The mold phase (25°C–30°C) is single oval conidia at the ends of short conidio- phores or directly on the hyphae.
c. The major endemic areas are south of the Ohio River and east of the Mississippi River.

3. Coccidioidomycosis is caused by *Coccidioides immitis* and is commonly known as “San Joaquin valley fever.” Transmission is via inhalation of the arthroconidia from sand and dirt. The primary respiratory infection rapidly disseminated in immunocompromised patients.
   a. The tissue phase (yeast) is large, thick-walled spherules filled with endospores.
   b. Alternating barrel-shaped arthroconidia between disjunctor cells are characteristic of the mold phase.
   c. The major endemic area is the San Joaquin Valley and the lower Sonoran Desert in the southwestern United States.
   d. Diagnosis is based on serologic and skin testing. In endemic areas, 3% of the normal population convert to skin-test positive every year.

4. South American Blastomycosis, or paracoccidioidomycosis, is caused by the systemic fungus *Paracoccidioides brasiliensis*. Transmission is via inhalation and primary infections are pulmonary or mucocutaneous. The prognosis is death within 2–3 years of dissemination of the disease.
   a. The diagnostic tissue phase is large, thick-walled yeast with multiple buds, resembling a “ship’s wheel.”
   b. Intercalary and terminal chlamydoconidia are observed in the mold phase.
   c. The endemic area is South America, especially in Brazil.

H. Superficial mycoses are noninvasive infections that affect the outermost layer of skin and hair.

1. Pityriasis versicolor is caused by *Malassezia furfur*, which infects the stratum corneum epidermis and causes hypopigmentation or hyperpigmentation on the trunk of the body. Scaly patches of skin fluoresce with a Wood’s lamp. Characteristic “sphagetti and meatballs” morphology is observed microscopically and a lipid oil overlay is required for culture.
   a. Black, yeasty colonies develop short olive-gray mycelia with age.
   b. Microscopically, dark septate hyphae with one- or two-celled blastoconidia are demonstrated in clusters along the hyphae. The fungus hydrolyzes casein.

3. Piedra is an infection of the hair shaft that produces hair breakage. Treatment is cutting or shaving infected hairs and use of topical antifungals. Infections may disseminate in immunocompromised individuals.
   a. *Trichosporon beigelli* is the causative agent of white piedra and forms soft, light brown nodules around the beard and mustache hairs. Microscopically, the fungus demonstrates hyaline hyphae with blastoconidia and arthroconidia on CMT 80 agar.
   b. Black piedra is caused by *Piedraia hortai*, which forms firmly attached hard, black nodules around the outside of scalp hairs. Microscopically, it produces dark thick-walled hyphae with swellings.

I. Cutaneous mycoses are typically caused by the dermatophytic fungi. Some cutaneous mycoses can be caused by *Candida* species. The cutaneous mycoses involve skin, hair, and nails. Infections may be acquired from animals (e.g., *Trichophyton rubrum*, *Microsporum canis*, *Trichophyton verrucosum*) or from humans (e.g., *Epidermophyton floccosum*, *Microsporum audouinii*). Diagnosis is made by microscopic examination of skin, hair, and nails with 10% KOH. Infections are commonly referred to as *tinea infections* or *dermatophytonoses*. “Ringworm” is another name for dermatophytic infections. Microscopically, the fungi demonstrate *microconidia* and *macroconidia*. Both conidia types or only one type may be demonstrated by a fungus. Diagnosis is made by macroscopic and microscopic observations of the fungi and the location of the infection on the body.
1. Tinea capitis (head) is a dermatophytic infection of the scalp.
   a. Epidemic tinea capitis occurs in children and is extremely contagious. It is caused by *M. audouinii*, which fluoresces under a Wood’s lamp. Microscopically, the fungus demonstrates rare irregular, rough-walled macroconidia with two to eight cells and rare club-shaped microconidia.
   b. Zoophilic tinea capitis occurs primarily in children. Transmission of infection is through animals. It is most commonly caused by *Microsporum canis* with few microconidia and numerous rough thick-walled, spindle-shaped macroconidia with 6 to 15 cells. *Trichophyton mentagrophytes* is also a causative agent of zoophilic tinea capitis. It demonstrates grape-like clusters of microconidia and numerous pencil-shaped macroconidia with five to eight cells.
   c. Black-dot tinea capitis is a chronic infection in adults caused by *Trichophyton tonsurans*. Rare macroconidia and numerous microconidia are observed microscopically.
   d. Favus (tinea favosa) is a severe tinea capitis caused by *Trichophyton schoenleinii*. Macroconidia are only observed if the fungus is grown on sterile rice grains.
   e. *M. gypseum* is transmitted via contact with contaminated soil is a causative agent of tinea capitis. Numerous rough, thin-walled, elliptical macroconidia with four to six cells are produced by the fungus. Few microconidia are produced.

2. Tinea barbae is an infection of the beard, neck, or face.
   a. The causative agent *Trichophyton verrucosum* requires thiamine for growth and microscopically produces rare three- to five-celled macroconidia with a rat-tail end.
   b. *T. mentagrophytes* causes tinea barbae.
   c. *Trichophyton rubrum* causes tinea barbae. It produces numerous club-shaped microconidia, which occur singly along the hyphae. Rare to numerous smooth-walled, pencil-shaped macroconidia with three to eight cells are also produced. This dermatophyte is characterized by a deep red reverse.

3. Tinea corporis is a dermatophytosis of the body which usually affects the inside skin folds. Candida species are often causative agents of this infection.
   a. *T. rubrum*
   b. *T. mentagrophytes*
   c. *T. violaceum* has characteristic violet colonies with a lavender reverse in 2–3 weeks. Few microconidia are produced on thiamine-enriched media, in addition to chlamydoconidia in chains. No macroconidia are produced.

4. Tinea cruris is a dermatophytosis of the groin caused by the following fungi:
   a. *Epidermophyton floccosum* is a dermatophyte that produces two- to four-celled, smooth, club-shaped macroconidia. These are produced singly or in clusters. No microconidia are produced by this fungus.
   b. *T. rubrum*
   c. *T. mentagrophytes*
   d. *Candida species*

5. Tinea pedis is an infection of the feet, commonly called athlete’s foot. It can be caused by the following fungi:
   a. *T. rubrum*
   b. *T. mentagrophytes* is the most common cause of athlete’s foot,
   c. *E. floccosum* causes epidemic tinea pedis.

J. Subcutaneous mycoses typically result from traumatic implantation (e.g., via thorn, splinter) of a fungus into the cutaneous and subcutaneous tissues. Subcutaneous fungi invade the tissue and lymph nodes, and chronic, nonhealing, hard, lumpy, crusted ulcerations are usually on the extremities. Endemic areas are the tropics, but distribution is worldwide. These infections are caused by hyaline and dematiaceous, as well as the fungus-like bacteria.

1. Sporotrichosis, or Rose Gardner’s disease, is caused by the dimorphic fungus *Sporothrix schenckii*. Infections can be lymphocutaneous, fixed, or pulmonary.
a. At 37°C, microscopic growth is a cigar-shaped budding yeast, commonly referred to as cigar bodies. These are observed in tissue. Conversion to the yeast form is necessary for identification.

b. At 25°C–30°C, the mycelial form of the fungus is a daisy head or floweret of microconidia, which is produced at the end of unbranched conidiophores on delicate hyphae.

c. *S. schenckii* is found on plant material, such as rose thorn, sphagnum moss, and timbers.

2. *Eumycotic mycetoma, or madura foot*, is a disease characterized by swelling, sinus tract formation, and the presence of sulfur granules usually in the extremities. There is little pain or bone destruction. It typically occurs in third world countries.

a. *Pseudoallescheria boydii*, the teleomorph or sexual stage of *Scedosporium apiospermum*, is the most common cause of mycetoma in the United States. Asexual conidia or single or small, clustered annelloconidia produced terminally on long or short annellophores. Sexual ascospores are contained asci within a large cleistothecia.

b. *Madurella species* most commonly cause mycetoma in Africa. An initial puncture or abrasion with contaminated material, usually on the feet, hands, and buttocks, progresses slowly to involve an entire extremity. Infected extremities are large and deformed.

3. *Chromoblastomycoses* begin with traumatic implantation of the spore in a limb. The disease produces hard, dry, cauliflower-like lesions. Lymphatic channels are blocked by drainage, causing elephantiasis, deformity and abnormal enlargement of the infected limb. Black dots, or sclerotic bodies, are observed in deep parts of the lesion. Secondary bacterial infections may be a complication of the disease. Infections are caused by the dematiaceous fungi *Phialophora, Fonsecaea*, and *Cladosporium apiospermum*, the most common cause of mycetoma in the United States. Asexual conidia or single or small, clustered annelloconidia produced terminally on long or short annellophores. Sexual ascospores are contained asci within a large cleistothecia.

4. *Phaeohyphomycosis* ranges from a superficial infection to one with deep-organ involvement. Causative agents include the *Phialophora*, *Exophiala*, and *Wangiella*, all of which produce slow-growing, black, yeasty colonies with a short mycelium. The dematiaceous *Phialophora* produce terminal balls of phialoconidia supported by phialides. *Exophiala* and *Wangiella* species demonstrate dark annelloconidia in clusters or terminal balls, respectively.

5. *Actinomycotic mycetoma* is caused by the anaerobic actinomycetes (fungus-like bacteria). Sulfur granules may be observed in tissues, with symptoms including exudates and painful bone involvement. Infections with the endogenous anaerobic oral flora result in “lumpy jaw” or pulmonary and thoracic infections due to aspiration into the lungs. The aerobic actinomycetes, *Nocardiia*, *Streptomyces*, and *Actinomadura*, cause mycotic-like infections. These organisms are differentiated according to biochemical test and growth requirements.

K. Diagnostic tests for identification of the systemic fungi include antigenic skin tests, serology, and DNA testing. Several test methods can be used for serologic detection of fungal antibodies. Empiric therapy is initiated with fungal infections. Antifungal agents, such as amphotericin B, fluconazole, and ketoconazole may be used for treatment. Fungal susceptibility testing can be performed in event of a failed response to empiric therapy. Fungal susceptibilities are usually performed in reference laboratories.

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### VIROLOGY

A. **Viruses (virions)** are the smallest known form of infectious disease-causing agents.

1. **Size.** Viruses are 1/10 the size of bacteria and 1/100 the size of eukaryotic cells. They cannot be observed with a light microscope.
2. Structure. Viruses of humans are composed of nucleic acid [deoxyribonucleic acid (DNA) or ribonucleic acid (RNA)].
   a. The nucleic acid is surrounded by a protein matrix referred to as the nucleocapsid. The capsid surrounds the nucleocapsid and is composed of structural units called capsomers. Virus particles are either “naked” or have a lipoprotein structure, the envelope, which surrounds the capsid.
   b. Symmetry can be helical, icosahedral (20-sided polygon), or complex.

3. Viral replication can occur only in living cells and involves many host-cell enzymes and functions. The steps of replication are as follows.
   a. Attachment or adsorption occurs when the virion comes in contact with the host cell.
   b. Penetration occurs when the virus passes through the plasma membrane.
      (1) Enveloped viruses penetrate via fusion.
      (2) Naked viruses can penetrate transversely. Pinocytosis is the process of pinching through the plasma membrane, which results in viruses entering within cytoplasmic vacuoles.
   c. Uncoating is typically mediated by cellular proteases and results in the separation of the capsid from the viral genome.
   d. Synthesis of early proteins. These proteins are involved in genome replication.
   e. Synthesis of late proteins. These proteins are used in the development of structural components of the virion.
   f. Assembly is the packaging of the new copies of the genome nucleic acid into the capsid proteins.
   g. Release or egress of the progeny virus is the final step. The lipid envelope is acquired along with the glycoproteins as the progeny viral nucleocapsid buds through the membrane.

B. Specimen collection, transport, and processing

1. Time of collection. Specimens for viral identification are best collected during the time of first presentation of symptoms.
   a. Virus titers (concentration of virus) are usually highest in the early part of the illness.
   b. Serologically, a fourfold rise in the antibody titer between acute and convalescent sera has been used to identify a particular infectious agent as the cause of a recent disease.

2. Viral culture. The type of viral illness and the disease symptoms influence the specimens of choice for viral culture. Viral specimens are typically divided into nonsterile sites and sterile sites.
   a. Nonsterile sites include the following:
      (1) Conjunctival
      (2) Skin (e.g., mouth, lips)
      (3) Vesicular
      (4) Nasal (aspirates or washes)
      (5) Throat/upper respiratory tract
      (6) Sputum
      (7) Urine
      (8) Genital (cervical, penile)
      (9) Stool/rectal swab
   b. Sterile sites include the following:
      (1) Autopsy
      (2) Biopsy
      (3) CSF
      (4) Fine-needle aspirate
      (5) Blood
      (6) Bronchial alveolar (wash)
      (7) Pleural fluid
3. The procedure for specimen collection is vital for recovery of the infectious virus.
   a. Specimens collected with a Dacron or rayon swab (cotton swabs are not recommended for collection of viral samples because they are toxic to viruses) must not dry out during transport. The swab can be placed in a modified Stuart transport medium for transport to the laboratory.
   b. Nonsterile specimens that are not liquid, vesicular fluid, fine needle aspirates, or tissue biopsy specimens should be collected into viral transport media, such as Hanks’ balanced salt solution, 0.2 M sucrose-phosphate, or 2% fetal calf serum with Eagle’s minimum essential medium. Some transport media contain the following ingredients:
      (1) Antibiotics to inhibit growth of normal bacterial or fungal flora
      (2) Gelatin media to stabilize protein
   c. Urine for viral culture should not be collected into containers with preservatives.
   d. Blood for viral culture should be collected into heparin or ethylenediamine tetraacetic acid tubes.
   e. Stool specimens for viral culture should be collected into a clean container.
4. All specimens submitted for viral recovery regardless of source should be transported at refrigerated temperatures (0 °C–4 °C).
5. If the inoculation of the specimen into tissue culture media will exceed 5 days, the specimen should be quickly frozen at −70 °C.
6. Tissue culture is the inoculation of patient specimens into cells to determine the growth of a viral agent.
   a. Cells derived directly from the donor (animal or human sources) are known as primary cultures.
      (1) Primary rabbit kidney is the first choice for herpes simplex viruses types 1 and 2.
      (2) Primary monkey kidney is the first choice for the following viruses:
         (a) Influenza types A and B
         (b) Parainfluenza viruses types 1, 2, and 3
         (c) Mumps
         (d) Measles
         (e) Rubella
         (f) Enteroviruses
      (3) Primary human embryonic kidney is the first choice for the following viruses:
         (a) Parainfluenza virus
         (b) Measles
         (c) Rubella
         (d) Adenoviruses
         (e) Rhinoviruses
         (f) Herpes simplex viruses types 1 and 2
         (g) Varicella zoster
   b. Cell lines are primary cultures that have been subcultured.
      (1) Cell lines that have at least 75% of the cells with a normal chromosome complement are referred to as diploid (e.g., diploid human fibroblast cell line). Diploid cell lines usually die after 30 to 50 passages. The choices for culturing cytomegalovirus (CMV) are WI-38, MRC-5, or IMR-90.
      (2) Cell lines that have a predominance of cells containing an abnormal number of chromosomes are designated as heteroploid. Typically, these cell lines are continuous, that is, they have an indefinite number of passages.
         (a) AS49 cells, which are derived from a human lung carcinoma, are used to culture adenoviruses and varicella zoster.
         (b) HEP-2 cells, which are derived from a human laryngeal carcinoma, are used to culture respiratory syncytial virus (RSV) and adenoviruses.
         (c) HeLa cells, which are derived from a human cervical adenocarcinoma, are used to culture RSV, rhinoviruses, and adenoviruses.
c. *Order of inoculation.* The diploid cell lines are inoculated first. The continuous cell lines are inoculated second. The primary cells are inoculated last.

d. *Viral adsorption* refers to the time point when the virus comes in contact with the tissue culture cells.
   (1) **Stationary adsorption** is the procedure of simply incubating the cell and virus for 30 to 60 minutes at 35°C.
   (2) **The roller drum method** refers to the gentle rotation of the culture tubes to enhance adsorption.
   (3) **The low-speed centrifugation method** requires that shell vials be centrifuged at 750 to 1,000 × g for 30 to 40 minutes at 25°C.

e. *Viral detection* in the culture cells depends on virus replication in the cells. Virus may be detected by observing a **characteristic cytopathogenic effect**, such as swelling or inclusions.

7. **Direct examination** of clinical specimens for the presence of viral pathogens can be done by immunohistochemical staining, nucleic acid hybridization, or solid-phase immunoassay.
   a. **Immunohistochemical staining** uses fixed or fresh specimens incubated with chemically labeled (fluorescein) or enzymatically labeled (peroxidase) antibodies to detect viral antigens.
   b. **Nucleic acid hybridization** involves the detection of viral DNA or RNA sequences in nucleic acid extracted from specimens.
      (1) **Polymerase chain reaction** is a technique that allows the viral genes to be amplified to enhance detection.
      (2) **The Western blot (dot blot)** is a technique that employs single-stranded, complementary nucleic acid probes for detection of human immunodeficiency virus (HIV) in the blood of seronegative individuals.
   c. **Solid-phase immunoassays** use antibodies and radioimmunoassay or enzyme-linked immunoassortment assay (ELISA) to detect viral antigens.

C. **DNA viruses**

1. The **naked DNA viruses** with an icosahedral symmetry are the *Parvoviridae, Papillomaviridae, Polyomaviridae, Adenoviridae, and Hepadnaviridae.*
   a. *Parvoviridae.* Parvovirus B19 is spread by close physical contact. It causes erythema infectiosum, or fifth disease.
   b. *Papillomaviridae* have two significant genera, papillomavirus and papovavirus.
      (1) **Human papillomavirus** causes warts in humans and is associated with some genital cancers.
   c. *Polyomaviridae* produces histologically diverse tumors in various parts of the body (JCV and BKV)
   d. *Adenoviridae* have more than 47 serotypes of adenovirus. This virus causes infection of the respiratory tract (nasopharyngitis); the eye (keratoconjunctivitis); and the intestines (e.g., diarrhea, vomiting).
   e. *Hepadnaviridae* have a tropism for infection of the liver.
      (1) **Hepatitis B virus** is also known as serum hepatitis and chronic hepatitis.
      (a) Transmission of the virus is through the parenteral route (e.g., intravenous drug abuse and contaminated blood products) and sexual intercourse.
      (2) **Hepatitis surface antigen (HBsAg)** is present in early disease; anti-HBsAg indicates immunity.

2. The **enveloped DNA virus** with icosahedral symmetry is the *Herpesviridae,* which has three medically significant subfamilies: *Alphaherpesvirinae, Betaherpesvirinae,* and *Gammaherpesvirinae.* All have the ability to become latent after the primary infection.
   a. Within the group of *Alphaherpesvirinae* are herpes simplex 1 (HSV-1), herpes simplex 2 (HSV-2), and varicella zoster virus.
      (1) **HSV-1** is usually seen on the lips or skin or as an eye lesion. It may be diagnosed by the Tzanck cell test.
CHAPTER 8 Clinical Parasitology, Mycology, and Virology

(2) HSV-2 affects the genital or lips area and is frequently transmitted sexually.
   (a) Neonatal herpes is a severe disease of the newborn caused by contact with the virus during passage through an infected birth canal.
   (b) HSV-2 disease can be primary or recurrent.

(3) Varicella zoster virus causes a primary disease (chickenpox) and a recurrent disease (shingles).
   (a) Chickenpox is a mild, self-limiting, highly infectious disease that occurs mainly in children.
   (b) Shingles (or zoster) is caused by the reactivation of varicella zoster virus that has been latent in the neurons. Severely painful vesicles form on the trunk area.

b. The significant viral pathogen in the Betaherpesvirinae is CMV.
   (1) CMV is typically an inapparent disease of childhood. In immunosuppressed patients, CMV can cause a generalized disease.
   (2) If CMV infection occurs in the uterus or soon after birth, it may cause fetal or infant death.

c. The viral pathogen in the Gammaherpesvirinae group is the Epstein-Barr virus (EBV). EBV may cause infectious mononucleosis (IM) and is associated with Burkitt's lymphoma.
   (1) IM, or "kissing disease," produces atypical lymphocytes and immunoglobulin (IgM) heterophile antibodies that are detected by the monospot test.
   (2) Burkitt's lymphoma patients have elevated titers of EBV antibodies.

d. The human herpes virus type 6 causes a common exanthem disease (roseola infantum), or sixth disease.

3. The Poxviridae are naked DNA viruses with a complex symmetry. They include the human viruses vaccinia, variola, and molluscum contagiosum.
   a. Vaccinia is a variant of the variola virus and produces a mild disease. Vaccinia is used as the immunogen in the smallpox vaccination.
   b. The variola virus is the causative agent of smallpox, a disease that the World Health Organization presumes to be eradicated.
   c. Molluscum contagiosum causes small wart-like lesions on the face, arms, buttocks, and genitals.
      (1) This virus can cause a disease that mimics genital herpes.
      (2) M. contagiosum forms eosinophilic inclusion bodies in infected cells.

D. RNA viruses

1. The enveloped RNA viruses with helical symmetry are the Orthomyxoviridae, Paramyxoviridae, Arenaviridae, Rhabdoviridae, Coronaviridae, and Bunyaviridae.
   a. The orthomyxoviruses have a hemagglutinin, a neuraminidase, and a matrix protein associated with the envelope.
      (1) The influenza viruses are classified as types A, B, and C.
         (a) Types A and B are responsible for the epidemics of respiratory tract infections.
         (b) Reye's syndrome is associated with influenza type B.
      (e) Influenza is diagnosed by viral isolation or the hemagglutination inhibition test.
      (2) Antigenic drift is caused by a minor mutation in the hemagglutinin glycoprotein that leads to yearly epidemics.
      (3) Antigenic shift is caused by a major shift in the hemagglutinin glycoprotein that leads to intermittent pandemics.
   b. Paramyxoviridae are divided into three genera: paramyxoviruses, morbilliviruses, and pneumoviruses.
      (1) The paramyxoviruses are associated with the parainfluenza and mumps viruses.
         (a) Parainfluenza virus causes croup in infants.
(b) The mumps virus results in a generalized disease associated with enlargement of the parotid glands.

(2) The morbillivirus is associated with the measles virus and causes a maculopapular rash, fever, and Koplik’s spots on the buccal mucosa.

(3) The pneumovirus responsible for bronchiolitis and pneumonia in infants is RSV. RSV is rapidly diagnosed by demonstration of viral antigens in nasal washings with fluorescent antibody or immunohistochimical techniques.

c. The Arenaviridae are not typically encountered in the United States. Included in this family are the hemorrhagic fevers (e.g., Junin, Machupo, and Lassa viruses) and the flu-like illness caused by the lymphocytic choriomeningitis virus.

d. Rhabdoviruses are bullet-shaped viruses associated with the human pathogenic rabies virus and bovine vesicular stomatitis virus (VSV).

(1) The rabies virus produces a fatal disease following inoculation by an animal bite or, occasionally, by inhalation.

(a) The rabies virus produces specific cytoplasmic inclusion bodies called Negri bodies in infected cells.

(b) Postexposure prophylaxis consists of passive immunization with human rabies immune globulin.

(c) Diagnosis is verified by biopsies from skin, cornea impressions, or post-mortem examination of brain tissue.

(2) VSV is a mild disease associated with cattle.

e. The coronaviruses are the second most frequent cause of the common cold and have been implicated in infant gastroenteritis. Severe acute respiratory syndrome is associated with SARS-CoV. These viruses are typically difficult to isolate in the early stages in the laboratory.

f. Members of the family Bunyaviridae are Hanta virus, LaCrosse virus, and California virus.

(1) The Hanta virus has been associated with severe life-threatening respiratory tract infections in the southwestern United States. Transmission has been through exposure to infected deer mice droppings.

(a) Thrombocytopenia

(b) Left shift to myelocytes or earlier stages

(c) Presence of blastic forms among lymphocytes

(d) Proteinuria

(2) The California and LaCrosse viruses produce encephalitis, mainly in the Mississippi and Ohio River Valleys. Transmission is via an infected mosquito bite.

2. The enveloped RNA viruses with icosahedral symmetry are Togaviridae, Flaviviridae, and Retroviridae.

a. The two medically significant genera in the Togaviridae are the alphavirus and the rubivirus.

(1) The alphaviruses are arboviruses with mosquito vectors and animal reservoirs, such as:

(a) Eastern equine encephalitis

(b) Western equine encephalitis

(c) Venezuelan equine encephalitis

(2) The significant rubivirus is the rubella virus, which causes German measles, a systemic infection characterized by lymphadenopathy and a morbilliform rash.

(a) The rubella virus can produce congenital infections that can cause severe birth defects.

(b) Diagnosis is by a serologic test for IgM antibodies.

b. The Flaviviridae include the dengue virus, hepatitis C, St. Louis encephalitis virus, yellow fever virus, and West Nile virus.
Dengue virus is an arbovirus transmitted by mosquitoes. 

Hepatitis C virus, also known as non-A, non-B virus, causes 90% of the hepatitis cases associated with blood transfusion or infected blood products. 

St. Louis encephalitis virus is an arbovirus transmitted by mosquitoes. 

Yellow fever virus is an arbovirus transmitted by mosquitoes. 

West Nile virus is transmitted by mosquitoes and associated with contaminated blood products, organs, and tissues.

c. The Retroviridae contain a reverse transcriptase enzyme—RNA-dependent DNA polymerase. This family is classified into three genera: lentiviruses, spumaviruses, and oncoviruses.

(1) The lentivirus genus contains HIV-1 and HIV-2, which are the causative agents of AIDS.

(a) Transmission is through sexual intercourse, intravenous drug use, or transplacental from mother to child.

(b) AIDS is preceded by AIDS-related complex, which is characterized by anorexia.

(c) Full-blown AIDS is characterized by Kaposi’s sarcoma, Pneumocystis carinii pneumonia, CMV, and AIDS-related dementia.

(d) Antibodies to HIV are detected by enzyme immunoassay (EIA) and confirmed by the Western Blot technique. ELISA: simple screening tool for the presence of HIV antibody. Western Blot: Patient, who is screen positive, must also demonstrate the bands of gp41, p24, and/or gp120/gp160. This combination is regarded as confirmation of the presence of HIV virus. Viral Load: is a more sensitive test that detects low quantities of HIV-1 RNA. CD4 Lymphocyte Counts: marker of disease progression.

(2) Human T-cell leukemia virus I (HTLV-I) is classified within the Type C oncoviruses.

(a) HTLV-I causes a neurologic degenerating disease.

(b) Transmission is the same as for HIV.

(c) Long-term effects include debilitation and paralysis.

3. The naked RNA viruses with icosahedral symmetry are Picornaviridae and Reoviridae.

a. The medically significant picornaviruses are classified as enteroviruses or rhinoviruses.

(1) The enteroviruses include the polioviruses, coxsackievirus types A and B, echoviruses, enteroviruses, and the hepatitis A virus.

(a) The poliovirus can cause a mild illness, aseptic meningitis, or poliomyelitis (poliovirus types 1, 2, or 3).

(i) Spinal cord motor neurons are killed, and flaccid paralysis results.

(ii) Diagnosis is through serologic testing or cultivation of the virus from throat culture or feces.

(b) Coxackievirus type A infections are associated with the following diseases: hand-foot-and-mouth disease, hemorrhagic conjunctivitis, aseptic meningitis, and colds.

(c) Coxackievirus type B infections are associated with the following diseases: herpangina, viral heart disease, and Bornholm disease (pleurodynia).

(d) Echovirus primarily infects the enteric tract but can cause a range of diseases from the common cold to meningitis and hemorrhagic conjunctivitis.

(e) Enterovirus infections are associated with respiratory tract infections, central nervous system disease, and hemorrhagic conjunctivitis.

(f) Hepatitis A virus (HAV) is also referred to as infectious hepatitis.

(i) Transmission is via the fecal-oral route.

(ii) Infections can cause epidemics.

(iii) HAV is diagnosed serologically by a rise in IgM as detected by ELISA.

(2) The rhinoviruses are the most frequent cause of the common cold. More than 100 serotypes exist.
The primary pathogen in the Reovirus group is the rotavirus, which is the primary cause of acute infantile diarrhea. Diagnosis is by demonstration of virus in the stool or by serologic testing (e.g., ELISA).

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CHAPTER 9
Urinalysis and Body Fluid Analysis

BARBARA SAWYER, PhD, MT (ASCP), CLS (NCA)

I. THE RENAL SYSTEM

A. Structure and function

1. Components. The urinary system consists of two bean-shaped kidneys, the ureters, which carry urine into the bladder for storage, and the urethra, which transports urine outside the body.

2. Kidneys. Each kidney weighs approximately 150 g and measures 5 cm by 12 cm.
   a. Anatomy
      (1) Each kidney contains 1 to 1.5 million nephrons, which are the functional units of the kidney. A nephron is composed of a glomerulus, which is the filtering unit, and renal tubules, which are 30 to 40 mm in length.
      (a) A glomerulus (plural: glomeruli) is made up of tufts of blood vessels formed from the afferent (entering) and efferent (exiting) arterioles.
      (b) The renal tubules include Bowman’s (glomerular) capsule, the proximal convoluted tubules, the loops of Henle, and the distal convoluted tubules.
      (2) The cortex of the kidney contains the glomeruli and the proximal convoluted tubules.
      (3) The medulla of the kidney consists of the loops of Henle, the distal convoluted tubules, and the collecting tubules.
      (4) The calyx is the area where the collecting tubules join together and empty freshly formed urine into the renal pelvis. From there, the urine flows into the ureters, then to the bladder, and out the urethra.
   b. Function. The kidneys maintain homeostasis by regulating fluid balance, acid-base balance, and electrolyte balance. They are primarily excreters of waste products and serve to maintain blood pressure and erythropoiesis.

B. Urine formation. In an adult, urine output volume ranges from 400 to 2,000 mL/day.

1. Overview. Approximately 1,200 mL of blood per minute (i.e., 20%–25% of blood volume) is supplied to the kidneys through the renal artery, which branches into the afferent arterioles and efferent arterioles.
   a. Glomerular filtration is accomplished through the thin walls of the afferent and efferent arterioles. The difference in the size of the lumen of these two vessels produces an increase in the hydrostatic pressure within them. This pressure increase forces the filtrate through the thin capillary epithelium and into the space of Bowman’s capsule, which surrounds the glomerulus (see Web Color Image 9–1).
A Concise Review of Clinical Laboratory Science

1. The glomerulus functions as a sieve or filter. The capillaries retain blood cells and serum proteins, whereas smaller molecules (ions, amino acids, glucose, urea, creatinine, uric acid, ammonia, or dissolved solutes with a molecular weight <70,000) and water filter into Bowman’s space.

2. Approximately 20% of the volume of plasma (i.e., 120 mL/min) that passes through the glomerular tuft is caught in Bowman’s space and is referred to as the glomerular filtrate. At this point, the filtrate is iso-osmotic with plasma and is called an ultrafiltrate. It has a specific gravity of 1.010 +/− 0.002 and a pH of 7.4.

3. The ultrafiltrate then passes into the tubular system, which consists of the proximal convoluted tubules, the loops of Henle, and the distal convoluted tubules.

b. Cellular transport mechanisms. Obviously, the body cannot lose 120 mL of water and essential substances every minute. Thus, when the ultrafiltrate enters the proximal convoluted tubules (PCT), cellular transport mechanisms begin to reabsorb essential substances and water. Cellular mechanisms involved in tubular reabsorption can be active or passive.

(1) Active transport occurs when substances to be reabsorbed combine with a carrier protein contained in the membranes of the renal tubular cells. Electrochemical energy produced by this interaction transfers the substance across the cell membrane back into the bloodstream. Substances reabsorbed by active transport include glucose, amino acids, and salts in the PCT, chloride in the ascending loop of Henle; and sodium in the distal convoluted tubules.

(2) Passive transport moves molecules across a membrane based on concentration gradient or electrical potential. The ascending loop of Henle is impermeable to water; therefore, passive reabsorption of water takes place in all other parts of the nephron. Urea is passively reabsorbed in the PCT and the ascending loop of Henle. Sodium is passively reabsorbed in the ascending loop of Henle.

C. Constituents of urine

1. Urine is continuously formed by the kidneys. Depending on dietary intake, physical activity, metabolism and endocrine function, concentrations of urine constituents vary.

   a. Urea accounts for half of the total dissolved solids in urine (6–18 g/24 h). It is a metabolic waste product from the breakdown of protein and amino acids in the liver.

   b. Other organic compounds in urine are creatinine (0.3–0.8 g/24 h) and uric acid (0.08–0.2 g/24 h). A fluid can be identified as urine if it contains a high concentration of urea and creatinine.

   c. Chloride (100–250 mEq/24 h) is the major inorganic solid dissolved in urine, followed by sodium (100–200 mEq/24 h) and potassium (50–70 mEq/24 h).

2. A small amount of protein, mainly albumin, is excreted (150 mg/d) in urine and urobilinogen is typically present at a concentration of 1 mg/dL (i.e., 1 Ehrlich unit [EU]).

3. In urinary sediment, a few squamous, transitional, and renal epithelial cells per high-power field (40X) as well as one to two red blood cells (RBCs) or one to five white blood cells (WBCs) are considered normal findings. Mucus and one to two hyaline casts per low-power field are common. Sperm cells are occasionally observed in a urine specimen from a female, but not a male. Amorphous urate and phosphate crystals, calcium oxalate, triple phosphate, and uric acid crystals are common findings in urine sediment.

II. THE URINE SPECIMEN

A. Routine urinalysis testing describes the results of a series of screening tests capable of detecting (in a semi-quantitative manner) renal, urinary tract, metabolic and systemic diseases. Urine is readily available and easy to collect.
CHAPTER 9 Urinalysis and Body Fluid Analysis

1. When there is disease of the kidney or bladder, kidney function may be impaired. Substances that are normally retained by the kidney may be excreted, and substances that are normally excreted may be retained. The routine urinalysis is a good screening test for the detection of changes in renal system.

2. Metabolic or systemic diseases may lead to the excretion of substances such as abnormal amounts of metabolic end products or substances specific for a particular disease that can be detected in urine. The amount of sodium or water that is excreted is also indicative of systemic or metabolic disease.

3. All body fluid specimens should be considered infectious and collected, transported, and handled according to safety protocols.

4. Urine specimens should be analyzed within 1 hour of collection, or they must be stored in a dark refrigerator between 4°C and 7°C to preserve chemical and cellular constituents.

B. Quality control in urinalysis

1. Quality control (QC) is a system for monitoring analytic testing to ensure the reliability and accuracy of each measurement performed on a specimen. Laboratory quality control procedures are designed and implemented to detect pre-analytical variables, such as improper specimen collection or inappropriate specimen type or preservation, and analytical variables including analytical (equipment or reagent problems) or technical errors during analysis to prevent reporting of incorrect individual results. By performing and adhering to QC procedures, a laboratory provides high quality service by removing the need for recollection and retesting.

2. Pre-analytical variables are monitored in the urinalysis (and body fluid analysis) laboratory by distributing prepared guidelines for specimen collection, required specimen types, and specimen preservation to all collection areas (e.g., physician’s offices, nursing stations, outpatient facilities, etc.). These guidelines must be evaluated periodically to ensure that they continue to meet the standards of laboratory accreditation and the needs of laboratory users.

3. Analytical variables in the urinalysis laboratory are monitored by analyzing control materials (i.e., substances with known concentration intervals) and comparing the observed values with the expected values published for the controls. The control material is analyzed exactly like the individual sample for the particular testing procedure. In urinalysis, much of the analysis is qualitative or semi-quantitative in nature and specific reference intervals are not used.

a. Numeric or qualitative limits are determined for each control. When tested, they must fall within the control limits to ensure that the analytic system is functioning properly.

b. Every testing method has a characteristic inherent variability. Thus, a control, if it is quantitative in nature, has a mean value ± two standard deviations, which is the 95% confidence limit of parametric statistics.

c. Each day that a method is performed, quality control material must be analyzed. This is a regular responsibility of clinical laboratory scientists. The quality control material can be a frozen pooled sample, commercially available lyophilized pooled material, or commercially available liquid preparations. It is important to follow the manufacturer’s instructions when preparing and using quality control materials.

(1) The control materials should be labeled with the date of preparation, its expiration date, and the initials of the person who prepared the control material.

(2) The control material is analyzed in the same manner that unknown samples are tested, but prior to the testing of unknown samples.

(3) Two to three different levels of control substances are usually tested.

(a) A normal control contains constituents at concentrations within healthy reference intervals or descriptions.

(b) An abnormal control contains analytic constituents at concentrations outside of the reference interval.

(4) A three-level control system usually contains a low, normal, and high reference range when medically significant decisions are made at each level.
The clinical laboratory scientist must assure that the control material has validated the testing procedure before any individual values are reported.

Control data should be permanently recorded with the date and name of the clinical laboratory scientist who has done the testing as well as an interpretation of the data. If any values are outside of the supplied reference intervals, controls should be re-run and equipment troubleshooting should begin. Any notes regarding these procedures must be kept with the permanent record.

4. Specific QC procedures. In a urinalysis laboratory, the first quality control measure is to assure the identity and proper collection, handling and preservation of the submitted specimens (pre-analytical variables). If there is no identifying label on the body of the urine container, the sample must be rejected. Collection, handling and preservation procedures must be followed according to set laboratory guidelines. Control of analytical variables includes the following procedures:

a. Proper performance of the refractometer (if used), reagent dipsticks for chemical testing, automated equipment, microscopes, and any confirmatory testing methods must be assessed and the results must be recorded. Specific protocols for testing the performance of equipment must be located in the laboratory procedure manual.

b. All controls and reagents have a lot number and expiration date. No reagents or controls are to be used past their expiration dates, even if they seem to be functioning properly. Any reagent that looks unusual or reacts outside of the reference or control interval should not be used. All lot numbers and expiration dates are recorded in the permanent quality control record.

c. Whenever a reagent is opened for the first time, the date and initials of the person placing the control or reagent in use must appear on the container.

d. Reagent dipsticks, which are plastic strips containing pads impregnated with chemicals for urine chemical testing, are subject to deterioration from moisture, heat, or light exposure. Reagent dipsticks are packaged in opaque containers that contain a desiccant. They should be stored at room temperature in a dry place and checked each day of use with at least two levels of a control material.

(1) Strips that are brown or discolored indicate that there has been contamination.

(2) The container should be recapped promptly after removing the number of strips needed for immediate testing.

(3) The reagent pad areas should not be touched.

(4) The strip should be dipped into a well-mixed specimen (control or individual) to moisten all test areas. The strip should be removed promptly, drawing it along the edge of the urine container to remove excess urine, and placing it horizontally on a clean piece of paper or in the automated strip reader. Excessive dipping or keeping the strip vertical can cause the reagents from one area to flow into another. If performed manually, the strip should be held close to the color chart on the container when the technician is reading results, and observations should be made in a well-lit area. If an automated strip reader is used, the manufacturer's directions for equipment use should be closely followed. All QC results of reagent strip tests should be maintained in a permanent record (hard copies or computer files) for a specified period of time.

(5) Whenever a new container of reagent strips or a new control is opened, the strips should be tested using the new control material. All lot numbers and expiration dates must be recorded.

(6) When testing unknown samples, any questionable reactions with the dipsticks should be checked with the confirmatory tests according to laboratory protocol.

(7) Interfering substances in the urine specimen or improper technique can lead to inaccurate results. The clinical laboratory scientist should be aware of the limitations and interfering substances for each dipstick reaction.

e. If a refractometer is used, the specific gravity of distilled water (reference range, 1.000 ± 0.001), 5% NaCl (1.022 ± 0.001), 3% NaCl (1.015 ± 0.001), 9% sucrose
(1.034 ± 0.001), or other known substances should be measured. The results should be recorded.

1. The specific gravity of a normal and abnormal control substance should be measured and the results recorded.

2. Verification should be made that the results are all in range and that the ranges are for that particular lot of control.

5. Whenever a confirmatory test is performed (e.g., a Clinitest for reducing substances, Ictotest for bilirubin, sulfosalicylic acid test for protein), the reagents must be checked with the normal and abnormal quality control material. Results must be recorded and verified as being within the reference interval before any unknown samples can be analyzed.

6. Commercially available control materials can be used to assess the microscopic portion of urinalysis testing. It is important to follow the manufacturer’s instructions regarding the procedure for preparing the control material to be microscopically assessed.

7. If a quality control result is out of control or out of the acceptable limits of the control range, the problem must be identified and corrected before any unknown testing can be performed.

8. The following steps are useful in identifying problems related to QC:
   a. A newly opened bottle of reagent should be tested with the controls. If the new reagent is acceptable, the old reagent can be discarded, and testing can continue with the new reagent.
   b. If the new reagent is also out of the control range, a new control can be opened. If the reactivity is now within range, the old control can be discarded, and testing can continue.
   c. If the reactivity is also out of the control range for the new reagent and the new control, the supervisor should be notified before the work is continued.
   d. All problems and resolutions should be noted in the permanent QC record of the laboratory manual.
   e. In general, potential inaccuracies that occur in urinalysis testing may be attributed to:
      (1) Failure to collect, handle, and preserve the specimen appropriately; failure to maintain the identity of a specimen throughout the testing phases
      (2) Failure to follow recommended procedures of the laboratory and manufacturer regarding reagents and control material; failure to observe expiration dates
      (3) Failure to use color charts for reagent dipstick and confirmatory testing
      (4) Failure to follow manufacturer’s instructions regarding use of automated equipment
      (5) Expecting tests based on different methodologies to agree
      (6) Lack of good control procedures

C. Types of urine specimens
   1. To assess a individual’s metabolic state, it is often necessary to regulate certain aspects of urine specimen collection, such as the time, length, and method of collection, as well as the individual’s dietary and medicinal intake.
   2. Types of urine specimens include: random, catheteric, first morning, and timed. A special “three-glass” urine specimen for prostate infection can also be performed. Urine specimens used for evaluation of drug presence must be collected in a special manner. Descriptions of these specimen types are listed below.

D. Methods of collection
   1. The random urine specimen is the most commonly encountered specimen and the most easily obtained.
      a. To obtain a random void specimen, the individual merely voids urine from his/her bladder into a container. The container, usually a 100 to 200 mL cup, should be sterile in case a bacterial, fungal, or viral culture may be needed. A random specimen may be collected at any time of the day and is an ideal specimen to screen for many abnormalities.
A random midstream “clean catch,” in which contamination from the external genitalia and vagina is minimized, is desirable if a bacterial culture is to be performed. Before this specimen is collected, the glans penis of the male or the urethral meatus of the female is thoroughly cleansed. To collect the specimen, the individual begins to void a few milliliters of urine into the toilet, then collects the midstream flow of urine into a sterile container. Any remaining urine is passed into the toilet.

e. A catheter specimen is obtained after a sterile catheter is inserted by a physician through the urethra into the bladder, which allows the urine to flow down the catheter into a collection bag. These specimens can be random or timed. They are used for routine testing and microbial cultures. Urethral catheterization is also used to examine the efficacy of a single kidney; dual-lumen urethral catheters retrieve a specimen from each kidney. A catheter is inserted through the urethra and into the ureter to obtain this type of specimen.

d. A suprapubic aspiration involves collecting urine directly from the bladder by puncturing the abdominal wall and entering the bladder with a sterile needle and syringe. The normally sterile urine is aspirated into the syringe and sent to the laboratory for analysis. This specimen can be used to diagnose bacterial infections of the bladder, especially anaerobes, as well as for cytology studies and routine testing. This technique may be used with infants to avoid fecal contamination of the specimen.

2. A first-morning specimen is collected immediately on arising and should be delivered to the laboratory as quickly as possible. It is used to evaluate orthostatic proteinuria and to detect low levels of substances that are difficult to observe in a random sample, such as hormones. Substances are more concentrated in a first morning specimen than in most random specimens because the urine is retained in the bladder overnight.

3. Timed collections enable quantitation of various analytes in urine. Circadian or diurnal variations in the excretion of various substances as well as the effect of exercise, metabolism, and hydration may necessitate collection of urine specimens at specific times, or for a specific amount of time (usually 2, 12, or 24 hours). Accurate timing is essential to ensure valid results. Depending on the substance being measured, a urine preservative, as well as dietary restrictions, may be necessary.

4. Drug-testing programs require particularly exacting methods of urine collection that follow a standard “chain-of-custody” process. This process ensures that no specimen corruption has occurred. Proper identification of the specimen must be maintained throughout the collection, transport, and testing process. Signatures of all who come in direct contact with the specimen must be placed on the chain-of-custody form. Typically, the urine specimen for drug testing is a random sample. Specimen collection may be observed to guarantee that the sample is not being tampered with. To assess possible tampering, urine temperature can be measured within a period of 4 minutes following collection and must fall between 32.5°C and 37.7°C to be considered acceptable; otherwise the specimen may have been altered. Because of the exacting requirements that must be followed in drug collection procedures, it is best to review all procedures at http://www.drugfree-workplace.gov/DrugTesting/DDItesting.aspx.

5. A so-called “three-glass” specimen involves collection of three separate urine samples from a male. Urine is examined particularly for the differential presence of white blood cells and bacteria in each of the three samples. The first sample is a control, the second midstream sample is used as a control for kidney and bladder infection, and the third component of the sample follows prostatic massage. The third “glass” will contain prostatic fluid, and in prostatic infection, will contain a white blood cell and bacterial count at least ten times that of the first control specimen. If the second sample contains bacteria from a kidney or bladder infection, the results of the third sample are invalid.

E. Specimen Preservation. Because the changes in the composition of unpreserved urine can alter the physical, chemical, and microscopic results of a urinalysis, specimens must be delivered to the laboratory promptly or be preserved using an approved method. Testing of urine must occur within 2 hours of collection.
III. PHYSICAL EXAMINATION OF URINE

A. Appearance (Table 9–1). Observations of urine color and clarity should always be performed on a well-mixed, uncentrifuged specimen.

1. Variations in color. Urine color can vary from colorless to black.

   a. Typical urine color. Throughout the day, an individual’s urine color can be a pale straw (almost colorless) or yellow-amber color to a dark amber color, depending on the concentration of substances and amount of water in the urine. High fluid intake results in hypotonic dilute urine, which is pale in color. Darker urine results

<table>
<thead>
<tr>
<th>Appearance</th>
<th>Cause</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colorless</td>
<td>Dilute urine</td>
<td>Polyuria; random specimen</td>
</tr>
<tr>
<td>Cloudy, Hazy</td>
<td>Phosphates</td>
<td>Nonpathologic</td>
</tr>
<tr>
<td></td>
<td>Carbonates</td>
<td>Nonpathologic</td>
</tr>
<tr>
<td></td>
<td>Urates, Uric Acid</td>
<td>Nonpathologic</td>
</tr>
<tr>
<td></td>
<td>Cells</td>
<td>Infection; Inflammation</td>
</tr>
<tr>
<td></td>
<td>Leukocytes</td>
<td>Glomerular damage; vascular injury</td>
</tr>
<tr>
<td></td>
<td>Erythrocytes</td>
<td>Nonpathologic</td>
</tr>
<tr>
<td></td>
<td>Squamous epithelial</td>
<td>Nonpathologic; catheterization</td>
</tr>
<tr>
<td></td>
<td>Transitional epithelial</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Renal tubular epithelial</td>
<td>Necrosis of tubules</td>
</tr>
<tr>
<td></td>
<td>Bacteria</td>
<td>Infection of upper or lower tract</td>
</tr>
<tr>
<td></td>
<td>Yeast</td>
<td>Vaginal contaminant</td>
</tr>
<tr>
<td></td>
<td>Spermatozoa</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mucus</td>
<td>Nonpathologic; catheterization</td>
</tr>
<tr>
<td></td>
<td>Abnormal crystals</td>
<td>Various metabolic and physiologic causes</td>
</tr>
<tr>
<td>Milky</td>
<td>Many neutrophils</td>
<td>Pyuria</td>
</tr>
<tr>
<td></td>
<td>Lipids</td>
<td>Possible nephritic syndrome</td>
</tr>
<tr>
<td>Straw, yellow</td>
<td>Dilute or normal urine</td>
<td>Random specimen</td>
</tr>
<tr>
<td></td>
<td>Diabetes insipidus</td>
<td>Polyuria</td>
</tr>
<tr>
<td></td>
<td>Diabetes mellitus</td>
<td>Polyauria</td>
</tr>
<tr>
<td>Dark yellow, amber</td>
<td>Concentrated urine</td>
<td>Dehydration; strenuous exercise</td>
</tr>
<tr>
<td></td>
<td></td>
<td>First morning specimen</td>
</tr>
<tr>
<td>Yellow-orange, orange</td>
<td>Bilirubin</td>
<td>Produces yellow foam</td>
</tr>
<tr>
<td></td>
<td>Acetilflavin</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Phenazopyridine (Pyridium)</td>
<td>Taken for urinary tract infections; can interfere with chemical test interpretation</td>
</tr>
<tr>
<td></td>
<td>Nitroantoin</td>
<td>Antibiotic</td>
</tr>
<tr>
<td>Yellow-green to yellow-brown</td>
<td>Bilirubin or biliverdin</td>
<td>Infection</td>
</tr>
<tr>
<td>Green to blue-green</td>
<td>Various medications</td>
<td>Antidepressants, muscle relaxants</td>
</tr>
<tr>
<td></td>
<td>Pseudomonas</td>
<td>Intestinal infection</td>
</tr>
<tr>
<td></td>
<td>Indican</td>
<td></td>
</tr>
<tr>
<td>Brown-black</td>
<td>Methemoglobin</td>
<td>Older urine; denatured hemoglobin</td>
</tr>
<tr>
<td></td>
<td>Homogeneous acid</td>
<td>Alkaptonuria</td>
</tr>
<tr>
<td></td>
<td>Melanin</td>
<td>Excess production by melanoma</td>
</tr>
<tr>
<td></td>
<td>Levadopa</td>
<td>Parkinson disease drug</td>
</tr>
<tr>
<td>Pink to Red</td>
<td>Erythrocytes (cloudy urine)</td>
<td>Glomerular damage; vascular injury; menstral contamination (clots)</td>
</tr>
<tr>
<td></td>
<td>Hemoglobin (clear urine)</td>
<td>Intravascular hemolysis</td>
</tr>
<tr>
<td></td>
<td>Myoglobin (clear urine)</td>
<td>Strenuous exercise; muscle injury</td>
</tr>
<tr>
<td></td>
<td>Porphyrins</td>
<td>Heme metabolites</td>
</tr>
<tr>
<td></td>
<td>Various foods</td>
<td>Breis, blackberries</td>
</tr>
<tr>
<td></td>
<td>Various medications</td>
<td></td>
</tr>
<tr>
<td>Red-purple</td>
<td>Porphyrins</td>
<td>“Port wine” color upon standing</td>
</tr>
</tbody>
</table>
from reduced fluid intake resulting in a darker, hypertonic urine. Urine color is produced by the presence of three pigments:
(1) Urochrome, which is a yellow pigment that is present in the highest concentration and is secreted by tubule cells;
(2) Uroerythrin, which is a red pigment
(3) Urobilin, which is an orange-red pigment from the oxidation of urobilinogen

b. Abnormal urine color may result from pathologic or nonpathologic conditions, such as medications and diet. Some dyes used to color food may cause changes in urine color, as can the ingestion of rhubarb and beets in genetically susceptible individuals.

(1) Red or red-brown is the most commonly seen abnormal urine color. A red color usually indicates the presence of blood (hematuria) or hemoglobin (hemoglobinuria).
   (a) If this color is seen in the urine of a female, contamination with menstrual blood should be considered.
   (b) Other causes of red-colored urine include myoglobin (myoglobinuria), porphyrins (porphyrinuria), food dyes, or ingestion of rhubarb or beets.

(2) Dark brown to black urine can be caused by alkaptonuria or malignant melanoma.
   (a) Alkaptonuria is a rare disorder caused by a lack of the enzyme homogentisic acid oxidase. This enzyme is required for the catabolism of tyrosine and phenylalanine. In this disorder, homogentisic acid is excreted into the urine. The urine is normal in color when freshly voided but turns black on standing or when alkalined.
   (b) Individuals who have malignant melanoma excrete melanogen into their urine. On exposure to light, this chromogen is converted to melanin, which is black.

(3) Yellow-brown to yellow-green urine results from the excretion of bilirubin (oxidized to biliverdin) or bile pigments into the urine of individuals who have obstructive jaundice.

(4) Colors ranging from green to blue typically indicate the presence of a drug metabolite or dye; however, the presence of indican (resulting from abnormal breakdown of tryptophan) will produce blue urine. Green urine may indicate a bacterial infection with Pseudomonas.

2. Urine appearance, transparency, clarity, turbidity or character indicates the presence of particulate matter. It is important to examine a urine specimen at room temperature for clarity.
   a. Urine is essentially clear, although cloudy turbid urine does not always indicate a pathologic condition.
   b. Turbidity is often caused by crystal precipitation, referred to as amorphous material, when a specimen is refrigerated. This is a nonpathologic cause of turbidity. Other nonpathologic causes of turbidity include the presence of mucus, powders, squamous epithelial cells, and spermatozoa (in women). Pathologic causes of turbid urine include the presence of bacteria, yeast, cells, abnormal crystals, spermatozoa (in men), prostatic fluid, or lipids.

B. Odor indications in a urine sample. Odor is typically not reported on a urinalysis report unless it is unusually prominent.

1. Normal odors. Urine has a faint, aromatic odor caused by the presence of volatile acids produced by the ingestion of various foods. Distinctive odors may result after ingestion of certain foods (e.g., asparagus, garlic, onions).

2. Abnormal odors may indicate pathologic conditions, improper handling, or improper storage of the urine specimen. If allowed to stand, especially unrefrigerated, bacteria in a urine specimen break down urea to form ammonia and a strong ammoniacal odor.
   a. Urinary tract infections. Large numbers of bacteria present in urine specimens from individuals who have urinary tract infections (UTIs) can result in a putrid, foul odor.
b. Ketone bodies. Increased ketone bodies excreted by diabetic or individuals who are on starvation diets give the urine a fruity odor.
c. Tubular necrosis. A lack of any odor may indicate acute tubular necrosis.
d. Amino acid disorders produce characteristic smelling urines. 
   (1) Phenylketonuria is associated with a mousy-smelling urine. 
   (2) Tyrosinuria produces a rancid odor. 
   (3) Maple syrup urine disease produces a maple syrup-smelling urine. 
   (4) Methionine malabsorption causes urine to smell like cabbage. 
   (5) Isovaleric acid and glutaric acid in urine give it a sweaty feet smell. 
   (6) Trimethylaminuria makes urine smell like rotting fish.

C. Specific gravity, refractive index, and osmolality measurements of urine indicate the amount of dissolved solids in urine or its concentration.

1. Specific gravity is a measure of the weight of a substance compared with an equal volume of pure, solute-free water at the same temperature. Urine is water that contains dissolved substances, primarily urea, sodium, and chloride. The specific gravity of urine is a measure of its density, and it is influenced by the number and size of the particles present. Because the specific gravity of water is 1.000, and the specific gravity of urine is compared with it, the number is a ratio and has no units. For urine, the specific gravity is reported to the third decimal place with the healthy reference interval from 1.003 to 1.035.

2. Specific gravity reflects the ability of the kidney to concentrate and dilute urine. Ordinarily, the specific gravity of urine is inversely proportional to the volume. With disease of the kidney, this ability is lost, and the specific gravity is fixed at 1.010 (similar to the initial plasma filtrate concentration of the glomerulus) and is termed isosthenuria. Urine specific gravity below 1.010 is termed hyposthenuric and above 1.010 is termed hypersthenuric.

3. There are three basic methods for determining the specific gravity of urine: by urinometer, refractometer, and reagent dipstick. The reagent strip is most commonly used. Harmonic oscillation is used in automated instruments to determine specific gravity (see Section VI below).

a. The urinometer is rarely used in a clinical laboratory and is included here only for completeness. It is a glass float weighted with mercury with an air bubble above the weight and a graduated stem on top. The weighted float displaces a volume of liquid equal to its weight and is calibrated at a specific temperature to sink to a level of 1.000 in distilled water. Dissolved substances in urine provide additional mass that causes the float to displace a smaller volume of urine than distilled water. The specific gravity measurement is read at the bottom of the meniscus on the stem of the urinometer. Urinometry is considered an inaccurate method.

b. A refractometer is not often used in a clinical laboratory. It measures the refractive index of a solution, which is the ratio of the velocity of light in air to the velocity of light in a solution. The velocity depends on the number of dissolved particles in the solution and determines the angle at which light passes through the solution. The clinical refractometer measures the angle and mathematically converts this angle to specific gravity, which is read from a scale in the handheld instrument. Refractometers should be calibrated each day of use. Distilled water and sodium chloride or sucrose solutions of known concentration, as well as commercial controls, should be measured and recorded.

(1) The major advantages of the refractometer method are: a small volume of specimen is required (1–2 drops); no temperature corrections are required; it is simple to operate; and it gives rapid, reliable results.

(2) Disadvantages include required corrections for large amounts of glucose and protein, as with the urinometer, and a scale reading maximum of 1.035. Very concentrated specimens or urine samples contaminated with radiographic dyes need to be diluted and remeasured.

c. Reagent strips can be used to measure the concentration of ions and give an indirect measure of specific gravity. The results are not identical to specific gravity
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because not all substances in urine ionize. Some ions, such as glucose, urea, and radiographic dyes, do not react with the reagent strip. The principle of the reagent strip reaction involves a change in the dissociation constant of a polyelectrolyte on the reagent pad. When dipped into urine, the polyelectrolyte on the pad releases hydrogen ions in proportion to the number of ions already in the solution; the more hydrogen ions released into the solution, the lower the pH, which reacts with an indicator dye on the reagent pad. Low specific gravity (few ions in the urine, few hydrogen ions released from the polyelectrolyte on the pad, alkaline) will produce a blue color, whereas a higher specific gravity (more ions in the urine, more hydrogen ions released from the pad, acid) produces a green to yellow color.

Interfering substances. Proteins will produce a false positive (higher specific gravity reading) because of anions present in the protein. Highly buffered alkaline urines decrease the value because the method contains an indicator that changes color when acid groups of the polyelectrolyte on the reagent strip dissociate in proportion to the number of ions in the urine.

The disadvantage of the reagent strip compared with the refractometer is that readings are in 0.005 intervals and must be compared to a color chart. The strips need to be checked each day of use with commercially available control urines and whenever a new container is opened.

4. Osmolality is a measure of the number of solute particles per unit of solvent. It is affected only by the number of particles present and not their density. It is used to assess the renal concentrating ability.

a. Evaluation. When evaluating renal concentration ability, small molecules (primarily sodium and chloride) are of interest. Urea is not of interest and contributes more to the specific gravity than do sodium and chloride. Because all three of these molecules contribute equally to the osmolality of a urine specimen, a more representative measure of renal concentrating ability can be obtained by measuring serum and urine osmolality.

b. Specific gravity and osmolality have a good correlation in nondisease states, but if an individual has a renal disease, osmolality measurements are preferred because high-molecular-weight substances (e.g., protein and sugar, which are excreted in various disease states) contribute to the specific gravity measurement.

c. Measurement of urine osmolality can be done using a vapor pressure osmometer or a freezing-point depression osmometer. These methods are described in Chapter 1, Clinical Chemistry.

D. Urine volume

1. Urine volume normally ranges from 400 to 2,000 mL per day in a healthy adult.

a. Oliguria is a decrease in the normal daily urine output and commonly accompanies states of dehydration, such as vomiting, diarrhea, perspiration, or severe burns.

b. Anuria is the cessation of urine flow and may result from serious damage to the kidneys or from a decrease in blood flow to the kidneys.

c. Nocturia is an increase in the nocturnal excretion of urine.

d. Polyuria is an increase in normal daily urine output and is observed in individuals who have diabetes mellitus and diabetes insipidus. This condition is also induced with use of diuretics, caffeine, or alcohol consumption.

IV. CHEMICAL EXAMINATION OF URINE

A. Chemical abnormalities detected in urine. Testing of a urine specimen should be done as soon as possible (within two hours) after collection. Chemicals and cellular elements are not stable at room temperature in an unpreserved specimen sitting in light (Table 9-2). The most common chemical abnormalities detected in urine are increased amounts of sugars (e.g., glucose, galactose), protein, RBCs, WBCs, bilirubin, ketones, and urobilinogen.

B. Reagent strip screening tests (Table 9-3)

1. Routine urinalysis includes initial testing with reagent multitest strips, which are
Table 9–2 Possible Changes in an Unpreserved Urine Specimen When Left at Room Temperature in Light

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Possible Change</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Red blood cells, white blood cells, epithelial cells, casts</td>
<td>Decrease</td>
<td>Rapid decomposition in alkaline and hypotonic specimens</td>
</tr>
<tr>
<td>Glucose</td>
<td>Decrease</td>
<td>Utilized by cells and bacteria</td>
</tr>
<tr>
<td>Ketones</td>
<td>Decrease</td>
<td>Volatilized or utilized</td>
</tr>
<tr>
<td>Bilirubin and urobilinogen</td>
<td>Decrease</td>
<td>Unstable in light and acid urines</td>
</tr>
<tr>
<td>Bacteria</td>
<td>Increase</td>
<td>Most enteric bacteria double every 20 minutes</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Can alter other urinary constituents:</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Produce peroxidase, which interferes with blood dipstick reaction</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Urea-splitting bacteria produce ammonia, which makes pH alkaline</td>
</tr>
<tr>
<td>pH</td>
<td>Increase</td>
<td>Urea-splitting bacteria produce ammonia, which makes pH alkaline and interferes with specific gravity and protein dipstick reaction, may cause precipitation of crystals</td>
</tr>
<tr>
<td>Protein</td>
<td>Increase</td>
<td>Bacterial proliferation</td>
</tr>
<tr>
<td>Nitrates</td>
<td>Decrease</td>
<td>Reduced to nitrates by bacteria</td>
</tr>
<tr>
<td>Trichomonad</td>
<td>Decrease</td>
<td>Become immobile or die; possible misidentification as white blood cells</td>
</tr>
<tr>
<td>Crystals</td>
<td>Increase</td>
<td>Precipitation enhanced by standing and cooling, which causes an increase in turbidity</td>
</tr>
</tbody>
</table>

plastic strips containing square pads impregnated with chemicals. Strips are available to test up to 12 analytes. Chemical strip testing typically includes tests for urine pH, specific gravity, blood, protein, glucose, ketones, bilirubin, urobilinogen, leukocyte esterase, and nitrite (Table 9–4).

2. Procedure. The reagent strip is dipped into the urine specimen, and, at the specified time for each test (determined by the manufacturer), the color reaction of each test area is either manually compared to a color chart or assessed by an automated urine analyzer (see Section VI below). If performed manually, it is important to read each test at the time stated by the manufacturer by comparing the reaction colors to the color chart. Highly pigmented urines may cause difficulty in reading the colored reactions. The reagents incorporated into each test area vary depending on the manufacturer. Test methodologies may vary with each lot of strips. Therefore, it is important to read the package information sheet enclosed with all reagents before using them. Knowledge of each test methodology is needed to evaluate results and to select confirmatory testing methods, if necessary.

3. Specific tests

a. pH. The pH value is a measure of the kidney’s ability to preserve normal hydrogen ion concentration as part of the renal system’s role in maintenance of acid-base balance. The tubular cells exchange hydrogen for sodium, and urine becomes acidic.

1. Test methodology involves test strips coated with substances that can function either as proton donors or proton acceptors and that absorb radiant energy differently in these two different forms. Indicators in the test area react to the different forms, changing color according to the hydrogen ion content of the urine.

2. The pH of urine in a normal healthy adult ranges from 4.6 to 8, depending on dietary intake. High protein intake results in acidic urine; a diet high in vegetables and fruits makes the urine more alkaline.

b. The specific gravity reagent test area method is based on the pK_a change of a pretreated polyelectrolyte (e.g., polymethylvinyl ether/maleic acid) in relation to ionic concentration, where pH is the negative logarithm of the hydrogen ion
### Table 9–3: Summary of the Reagents, Test Principle, Sensitivity, and Factors That Can Affect Reagent Strip Testing

<table>
<thead>
<tr>
<th>Test Reagents</th>
<th>Principle</th>
<th>Sensitivity</th>
<th>Affecting Factors</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH indicators: methyl red and bromthymol blue</td>
<td>Substances act as either proton acceptors or proton donors</td>
<td>5–9</td>
<td>Bacterial growth and metabolism may cause marked increase</td>
</tr>
<tr>
<td>Specific gravity</td>
<td>Polyelectrolyte, bromthymol blue indicators, buffers</td>
<td>pKa change of pretreated polyelectrolyte</td>
<td>0.005 increments between 1.000 and 1.030</td>
</tr>
<tr>
<td>Blood</td>
<td>H₂O₂, tetramethylbenzidine, or orthotoluidine</td>
<td>Catalase and peroxidase activity of hemoglobin, myoglobin, and red blood cells causing oxidation of chromogen</td>
<td>5–20 red blood cells; 0.05–0.3 mg/dL hemoglobin</td>
</tr>
<tr>
<td>Protein</td>
<td>Citrate buffer at pH 3, tetrabromphenol blue indicator</td>
<td>“Protein error” of indicators, indicator combines with protein, which alters its spectral absorption</td>
<td>5–20 mg/dL albumin</td>
</tr>
<tr>
<td>Glucose oxidase; peroxidase; o-toluidine, potassium iodide, or aminopropylcarbazol</td>
<td>Glucose + O₂ → gluconic acid + H₂O₂; H₂O₂ + chromogen → oxidized chromogen + H₂O</td>
<td>0.1 g/dL; more sensitive than Clinitest</td>
<td>≥50 mg/dL ascorbic acid may inhibit reaction</td>
</tr>
<tr>
<td>Ketones</td>
<td>Sodium nitroprusside</td>
<td>Acetoacetic acid + sodium nitroprusside → purple</td>
<td>5–10 mg/dL</td>
</tr>
<tr>
<td>Bilirubin</td>
<td>Dichloroaniline or dichloro-benzene-diazonium tetrafluoroborate</td>
<td>Diazo reaction: bilirubin + diazonium salt → tan to purple</td>
<td>0.4–0.8 mg/dL; less sensitive than Ictotest</td>
</tr>
<tr>
<td>Urobilinogen</td>
<td>Dimethylaminobenzaldehyde</td>
<td>Urobilinogen + dimethylaminobenzaldehyde → yellow to brown-orange</td>
<td>0.2–8 Ehrlich units</td>
</tr>
<tr>
<td>Leukocyte esterase</td>
<td>Indoxylcarbonic acid ester, diazoum salt</td>
<td>Indoxylcarbonic acid ester + leukocyte esterases → indoxyl + diazoum salt + purple</td>
<td>5–15/µL intact and lyed leukocytes with esterases</td>
</tr>
<tr>
<td>Nitrite</td>
<td>Para-arsanilic acid, tetrahydrobenzo(b)-quinolin-3-ol</td>
<td>Nitrite + para-arsanilic acid → diazounim + tetra-hydrobenzo(b)-quinolin-3-ol → pink</td>
<td>≥10¹⁸ bacteria/mL</td>
</tr>
</tbody>
</table>

H₂O₂ = hydrogen peroxide; mg/dL = milligrams per deciliter; µ/L = micrograms per liter; pKa = negative log of dissociation constant.

Concentration, and the pKₐ is the negative dissociation constant Kₐ of H₂CO₃. An increased concentration of electrolytes or many ions in urine decreases the pKₐ of the polyelectrolytes in the test area, causing a decrease in pH. The change in pH caused by increasing ion concentration is related to an increase in specific gravity. Specific gravity is an indirect measure of solutes in urine.

1. An increased specific gravity can be caused by the presence of glucose or protein in the urine. Radiographic dyes do not affect the dipstick method.
2. Persons with diabetes mellitus who are spilling glucose into their urine often have polyuria, which leads to a pale-colored urine with a high specific gravity.
3. Diabetes insipidus is a condition in which there is insufficient antidiuretic hormone (ADH). This condition is also accompanied by polyuria and pale urine, but the specific gravity is low.
Table 9–4 Clinical Significance of Chemical Dipstick Reactions

<table>
<thead>
<tr>
<th>Test</th>
<th>Clinical Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>Kidney’s ability to excrete excess acid, distal tubular dysfunction; acidic with</td>
</tr>
<tr>
<td></td>
<td>persistent metabolic and respiratory acidosis; persistent alkalinity with</td>
</tr>
<tr>
<td></td>
<td>metabolic alkalosis and urinary tract infection.</td>
</tr>
<tr>
<td>Specific gravity</td>
<td>Diabetes insipidus (deficient antidiuretic hormone); isosthenuria (loss of tubular</td>
</tr>
<tr>
<td></td>
<td>concentrating ability)</td>
</tr>
<tr>
<td>Blood</td>
<td>Bleeding in the urogenital tract as the result of trauma or irritations, such as</td>
</tr>
<tr>
<td></td>
<td>cystitis (bladder infection), glomerulonephritis (inflammation of the glomerulus),</td>
</tr>
<tr>
<td></td>
<td>pyelonephritis (inflammation of the kidney), burns, tumors, or exposure to toxic</td>
</tr>
<tr>
<td></td>
<td>chemicals; transfusion reactions; menstruation; myoglobin</td>
</tr>
<tr>
<td>Protein</td>
<td>Glomerular membrane damage, defective tubular reabsorption, immunoglobulin light</td>
</tr>
<tr>
<td></td>
<td>chains in multiple myeloma (Bence Jones protein), diabetic nephropathy; transient</td>
</tr>
<tr>
<td></td>
<td>elevation with fever, exercise, dehydration, acute phase of illness, pregnancy, and</td>
</tr>
<tr>
<td></td>
<td>orthostatic or postural proteinuria following long periods of standing</td>
</tr>
<tr>
<td>Glucose</td>
<td>Diabetes mellitus, pregnancy, impaired tubular reabsorption</td>
</tr>
<tr>
<td>Ketones</td>
<td>Diabetes mellitus, starvation diets</td>
</tr>
<tr>
<td>Bilirubin</td>
<td>Liver damage such as hepatitis or cirrhosis; obstruction of the bile duct</td>
</tr>
<tr>
<td>Urobilinogen</td>
<td>Liver damage such as hepatitis or cirrhosis; red blood cell lysis, porphyrimuria</td>
</tr>
<tr>
<td>Leukocytes</td>
<td>Urinary tract infections: cystitis, pyelonephritis</td>
</tr>
<tr>
<td>Esterase</td>
<td></td>
</tr>
<tr>
<td>Nitrite</td>
<td>Cystitis, pyelonephritis</td>
</tr>
</tbody>
</table>

(3) A fixed specific gravity of 1.010 (isosthenuria) indicates a loss of the concentrating ability of the glomerulus.

e. **Blood** in the urine may be in the form of intact RBCs or hemoglobin from lysed RBCs. Myoglobin will also react with the blood test area on the reagent strip.

(1) The dipstick method is based on the catalase and peroxidase activity of heme, myoglobin, and red blood cells, which cause oxidation of orthotoluidine or tetrabromophenol blue and produces a green to dark-blue color.

(2) The dipstick sensitivity is 5 to 20 RBCs, or 0.05 to 0.3 mg/dL hemoglobin and should normally be negative.

d. **Protein** is normally excreted into the urine at a concentration of 150 mg/24 h. Approximately one third is albumin, with the remainder being small globulins with molecular weights <50,000.

(1) The test is based on the “protein error” of indicators. The strip contains a citrate buffer at a pH of 3 and a tetrabromophenol blue indicator. At this pH, the indicator is yellow. Proteins present in the sample combine with the indicator, altering the spectral absorption of the dye; a yellow-green, green, or blue color results, depending on the concentration of protein.

(2) The test is specific for albumin but does not detect larger proteins.

(3) Highly buffered alkaline urine specimens may produce a false-positive result with this method.

e. **Glucose** is not typically detectable in urine. It is excreted into the urine when the plasma level exceeds the kidney threshold of 150 to 180 mg/dL or when there is a defect in the reabsorption mechanism of glucose.

(1) The test is specific for glucose. The sensitivity is 100 mg/dL.

(2) In the first of a double sequential enzyme reaction, gluconic acid and hydrogen peroxidase are formed from the oxidation of glucose by glucose oxidase. In the second reaction, peroxidase catalyzes the reaction of hydrogen peroxidase with potassium iodide chromogen, which produces color from blue to green, orange, or brown, depending on the quantity of glucose in the specimen.

f. **Ketones** are the products of incomplete fat metabolism, and their presence in urine indicates acidosis. The three ketone bodies present in urine are acetoacetic (diacetic) acid, acetone, and 3-hydroxybutyrate.
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(1) The test method is based on the reaction of sodium nitroprusside with acetoacetic acid. The test does not detect acetone or 3-hydroxybutyrate, but if one ketone is excreted, all are excreted. Positive reactions produce a maroon color.

(2) The test sensitivity is 5 to 10 mg/dL of acetoacetic acid.

g. Bilirubin is not normally detectable in urine. The reagent strip method can detect 0.4 to 0.8 mg/dL bilirubin with the diazo reaction, in which 2,4 dichloroaniline diazonium salt reacts with bilirubin to produce a tan-to-purple color.

h. Urobilinogen is normally present in urine in small amounts up to 4 mg/24 h.

(1) Paradimethylaminobenzaldehyde (Ehrlich’s reagent) reacts with urobilinogen (and porphobilinogen, depending on the brand of reagent strips) to form colors ranging from light yellow to brown-orange.

(2) In a random urine specimen, the normal concentration on the test strip reflects 0.2 and 1.0 Ehrlich units (EU). Increased concentrations can be detected by the strip method, but the method is not suitable for detecting decreases in concentration. Fresh specimens should be used for testing because urobilinogen is unstable in urine.

i. Leukocytes can be detected in urine with the leukocyte esterase reaction. Granulocytic leukocyte esterases catalyze the hydrolysis of the derivative pyrrole-N-tosyl-L-alanine ester to form pyrrole alcohol. The alcohol then reacts with a diazonium salt to produce a purple color.

(1) The test strip can detect 5 to 15 leukocytes per high-power field and is even sensitive to lysed granulocytes.

(2) Vaginal cellular contamination or trichomonads can cause a false-positive result.

j. The nitrite test provides a rapid screen for the detection of bacteria that are capable of reducing nitrates to nitrite. The test results should be negative.

(1) False-negative reactions may occur if:
   (a) The bacteria in the specimen do not have the enzymes necessary to reduce nitrates.
   (b) The diet of the individual is deficient in nitrates.
   (c) The bacteria reduce the nitrate beyond the nitrate state to nitrogen or ammonia.
   (d) The urine has not been in the bladder at least 4 hours, so the bacteria did not yet reduce the nitrates.

(2) On the reagent strip reaction, the Griess reaction detects nitrates that react with an aromatic amine at an acid pH. The resultant azo product reacts with a hydrobenzoquinolin compound to form a pink color in the presence of nitrite.

C. Confirmatory Testing

1. Glucose and other reducing substances, such as other disaccharides (e.g., fructose, galactose, lactose, pentose) and others (e.g., ascorbic acid) in the urine can be detected with the copper reductase test (Clinitest).

   a. The test uses the reagents copper sulfate, sodium hydroxide, sodium carbonate, and citric acid incorporated into a tablet.

   b. In the five-drop method, five drops of urine are added to 10 drops of distilled water in a test tube. One Clinitest tablet is added, and the solution begins to boil. Copper sulfate reacts with reducing substances in urine, converting cupric ions to cuprous ions in an exothermic process.

   (1) After 15 seconds, the tube is gently mixed and compared to a color chart. A negative reaction is blue. Depending on the concentration of reducing substance(s), the solution changes from green-blue to orange.

   (2) It is important to watch the reaction because if a high glucose concentration is present (>2 g/dL), the color reaction will pass from orange to a brownish color and back to blue (“pass-through” phenomenon). If this occurs, the urine must be retested at a lower concentration with a two-drop method (same procedure as the five-drop method, except two drops of urine are added to the test tube).
c. The test sensitivity is 150 mg/dL, which is less sensitive than the dipstick method. Thus, at low glucose concentrations, it is possible to have a positive dipstick test and a negative Clinitest. (1) It is also possible to have a positive Clinitest and a negative dipstick test if the sugar present is one other than glucose, or the urine contains another reducing substance (e.g., ascorbic acid).

   a. Nonglucose mellituria can occur in certain conditions. Inherited enzyme deficiencies can cause the accumulation of lactose, fructose, galactose, pentose, or sucrose in plasma. All of the sugar cannot be reabsorbed, and it appears in urine.

   b. Lactosuria can occur during late pregnancy or during lactation.

   c. Pentosuria can occur following ingestion of certain fruits.

   d. Fructosuria can occur with parenteral feedings with fructose.

(2) Sucrose is not a reducing sugar, so it does not react in the Clinitest.

2. Precipitation tests for protein are more sensitive than are the reagent strip methods and detect other proteins besides albumin.

   a. An aliquot (usually 1 mL) of 3% sulfosalicylic acid or trichloroacetic acid is added to an equal amount of urine in a test tube and mixed by inversion. The tube is allowed to stand for 10 minutes and then is inverted twice.

   b. The degree of precipitation is graded according to the following criteria:
      (1) Negative: no turbidity, ≤5 mg/dL protein
      (2) 1+: faint turbidity but no discrete granulation; ≈50 mg/dL protein
      (3) 2+: turbidity with granulation but no flocculation; ≈200 mg/dL protein
      (4) 3+: turbidity with granulation and flocculation; ≈500 mg/dL protein
      (5) 4+: clumps of precipitation; ≥1.0 g/dL protein

   c. The test sensitivity is 5 to 10 mg/dL and detects albumin, globulins, glycoproteins, and Bence Jones protein. Radiographic dyes react with this method. When the radiographic dyes are present, the specific gravity of the urine is usually >1.035, and typical crystals are seen on the microscopic examination of the urine specimen.

3. The Ictotest® is a sensitive test for the detection of bilirubin in urine in which 2,4-dichloro-benzene-diazonium tetrachlorozincate, sodium bicarbonate, and sulfosalicylic acid are combined into a tablet.

   a. Five drops of urine are placed on an asbestos cellulose test pad, which concentrates the specimen so that much smaller concentrations of bilirubin (0.05–0.1 mg/dL) can be detected as compared with the dipstick method.

   b. The urine is allowed to absorb into the pad for 1 minute, and then a reagent tablet is placed on the wet area of the pad.

   c. One drop of distilled water is placed onto the tablet. After 5 seconds, a second drop of distilled water is placed onto the tablet so that the drops run down the side of the tablet onto the test pad.

   d. The tablet is removed, and the test pad is observed within 1 minute for a purple color, which indicates the presence of bilirubin. A pink or red color is a negative reaction.

V. MICROSCOPIC EXAMINATION OF THE URINE

A. Urine sediment preparation

1. To ensure the accuracy and reproducibility of the urine microscopic examination, each laboratory must establish a protocol for the preparation of the urine sediment. Bright-field microscopy is the most commonly used method of sediment examination. To see sediments with a low refractive index, such as hyaline casts, sediment should be examined with decreased light obtained by decreasing the illumination, not by lowering the condenser. Phase-contrast microscopy is used to produce a phase difference of wavelengths of light, which gives better contrast when viewing low refractive index sediments (hyaline and cellular casts, mucous threads, and Trichomonas). Polarizing
microscopy involves the use of polarizing filters for observation of sediments that can rotate and refract light in two dimensions at 90° to each other (e.g., lipids and crystals).

2. Factors that must be considered in urinalysis to help standardize the microscopic examination include the following:
   a. Appropriate specimen collection, preservation, and handling. It is best to examine a specimen within 1 hour of individual voiding because many aspects of the chemical and microscopic examination change as the urine stands, especially at room temperature.
   b. A standard amount of urine, usually 12 mL, is placed in a conical tube and centrifuged for a uniform amount of time and speed, typically 5 minutes at a relative centrifugal force of 400.
   c. The spun tube is decanted, which leaves approximately 1 mL of fluid in which to resuspend the urinary sediment. If a stain is used, it should be added at this time.
   d. A small drop of well-mixed sediment is placed onto a microscope slide and shielded with a coverslip. Slides with uniform wells and coverslips are commercially available for the microscopic examination of urine. Drop size determines the amount of sediment viewed.
   e. A consistent method should be used to examine the urinary sediment. The slide is first viewed on low power (10×) to assess the overall composition of the specimen and to observe and count casts. Ten fields are examined at this magnification. The light must be very low to see hyaline casts. Other elements are counted on high power (40×). An average count of sediments in ten fields is usually reported.
   f. Microscopic results must be correlated with the color, appearance, and dipstick reactions for each specimen.

3. Microscopic sediment stains (supravital stains) can be used to aid the identification of formed elements.
   a. The most commonly used stain is the Sternheimer-Malbin stain, which consists of crystal violet and safranin O. This stain provides a more detailed visualization of the internal structure of cells and casts.
   b. Another supravital urine sediment stain is 0.5% toluidine blue. It differentially stains various cell components (e.g., the nucleus, cytoplasm) to help distinguish cells that may be similar in size, such as leukocytes and renal cells.
   c. Sudan III or oil red O stains are used to confirm the presence of neutral lipids. Lipids or fats within renal cells or histiocytes (oval fat bodies) or free-floating triglycerides stain red or orange with these two stains.
   d. The Prussian blue stain is used to confirm the presence of hemosiderin (iron) in epithelial cells and casts, as well as hemosiderin that is free-floating. The iron turns a characteristic blue color.
   e. Hansel stain consists of methylene blue and eosin-Y in methanol. It is used to identify eosinophils.
   f. One to two drops of 2% acetic acid added to a few drops of urine sediment can be used to differentiate RBCs from yeast cells, small WBCs, or epithelial cells. The RBCs lyse; the yeast cells remain intact. The internal structures of WBCs and the epithelial cells are accentuated.

B. Normal and abnormal cells in urine (Figure 9–1)

1. RBCs are small biconcave disks without a nucleus. They are 7–10 μm in diameter. In a hypertonic urine they become crenated, appearing to have a crinkled border. In a hypotonic, alkaline urine, the RBCs swell and may lyse. These lysed cell membranes are called “ghost” or “shadow cells” and appear as faint, colorless circles. Normally, a urine specimen can contain 0 to 3 RBCs per high-power field; increased numbers may indicate renal bleeding or glomerulonephritis (see Web Color Image 9–2).

2. WBCs, usually neutrophils, are larger than RBCs (10–15 μm diameter and contain a distinct nucleus. A normal urine sample contains 0 to 5 WBCs per high-power field. An increase is called pyuria and indicates the presence of an infection or inflammation in the genitourinary tract. Frequent causes of pyuria include bacterial infections (e.g., cystitis, pyelonephritis, prostatitis, urethritis) or nonbacterial disor-
3. There are three types of epithelial cells found in urine: squamous, transitional, and renal tubular. They are derived from the linings of the urogenital tract. A few of each type can normally be found in urine because of normal sloughing of old cells.

a. **Squamous cells** are derived from the lining of the vaginal tract and lower portions of the female and male urethras. They are the most frequently seen but least significant epithelial cell. Increased numbers in a female urine specimen indicate that the specimen was not collected using the midstream clean-catch technique. Squamous cells are large, 30 to 40 μm in diameter, and contain abundant cytoplasm with a small (7 μm in diameter) centrally located nucleus. Their cytoplasmic borders are irregular, and they are often folded over on themselves in a urine specimen. Squamous epithelial cells covered with bacteria are called “clue cells” and represent a vaginal bacterial infection (see Web Color Image 9–5).

b. **Transitional or caudate epithelial cells** line the urinary tract from the renal pelvis to the proximal two thirds of the urethra. They measure 12 to 20 μm and are characteristically round or pear-shaped with a centrally located nucleus. Unless present in large numbers (>10 per high-power field) with unusual morphology, transitional cells are seldom pathologic. Catheterization often causes these cells to appear in urine. When unusual in morphology or in large numbers, samples of these cells should be referred for cytologic examination and may indicate renal transplant rejection, acute tubular necrosis, ischemic injury to the kidney, or renal carcinoma (see Web Color Image 9–6).

c. **Renal tubular epithelial cells** line each portion of the renal tubules and are considered the most clinically important. Cells from the proximal or distal convoluted tubules are relatively large (20–60 μm). They are oblong or round to oval and contain an eccentric nucleus. Renal tubular cells from the collecting ducts range from 12 to 20 μm and are cuboidal, polygonal, or columnar. They have a single, large, dense nucleus that takes up approximately two thirds of its interior. Increased numbers in urine are the result of acute tubular necrosis from heavy metals or drug
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toxicity. Large numbers in urine are also caused by all types of renal diseases and are often accompanied by granular, waxy, or renal tubular cell casts and an increased number of blood cells (see Web Color Image 9–7).

d. Renal tubular cells containing fat are called oval fat bodies. They can be stained with Sudan III or oil red O. When visualized with polarized light, these fat globules display a Maltese cross shape. Oval fat bodies often indicate glomerular dysfunction with renal tubular cell death. When present in a urine specimen, these cells are accompanied by increased amounts of protein and cast formation (see Web Color Image 9–8).

4. Tumor cells, platelets, or epithelial cells with viral inclusions are rarely found in urine sediment. Cytologic techniques are more sensitive than conventional urine microscopy in detecting and classifying these kinds of cells.

C. Urine casts (Figure 9–2)

1. Urinary casts are formed in the distal and collecting tubules. Except for a few hyaline or granular casts, which can accompany strenuous exercise (athletic pseudonephritis) or severe stress, casts are not normally present in the urine. The presence of urinary casts is termed cylindroiduria and their appearance is often accompanied by proteinuria. Acid pH, urinary stasis, elevated protein, and concentrated solutes in urine all favor the formation of casts. Renal disease or damage along with these factors will produce different types of urinary casts. Casts are better identified with the use of a supravital stain and are typically reported as number of casts per low power field through the microscope.

2. Tamm-Horsfall protein, which is a mucoprotein secreted only by renal tubular cells, forms the matrix of casts. As the tubular lumen contents become concentrated (often due to stasis of urine flow), Tamm-Horsfall protein forms fibrils that attach it to ductal cells and hold it temporarily in place. As it is held in the tubule, it enmeshes into its matrix any cellular or chemical substance that is present in the filtrate at the time it is formed. Eventually, the cast detaches from the tubular epithelial cells and is flushed into the urine.

3. Because casts form in the tubules, they are cylindrical with parallel sides and rounded ends. Casts formed in the collecting ducts are broader than those formed in the proximal and distal convoluted tubules.

4. The number and type of casts reflect the extent of renal tubule involvement in disease processes. They are classified by the composition of their matrix and the type of substance enmeshed within them. Hyaline casts consist primarily of a homogeneous Tamm-Horsfall protein matrix with a low refractive index similar to urine. Also, they are the hardest to view because they do not contain any inclusions; they must be viewed with subdued light when using bright-field microscopy (see Web Color Image 9–9). These casts appear in urine after strenuous exercise or stress, although in small numbers they are considered as normal sediment.

5. Cellular casts consist of a matrix of protein covered with different cell types. Red blood cell casts are reddish in color and signify glomerular disease or physical damage to the glomerulus. The outline of an erythrocyte must be observed in part of the cast to identify these (see Web Color Image 9–10). White blood cell (leukocyte) casts are associated with pyelonephritis and infection. The white blood cells are larger than red cells and have multilobed nuclei and granules in the cytoplasm (see Web Color Image 9–11). Renal tubular epithelial cell casts are noted in tubular diseases such as drug toxicity and tubular necrosis; the cells in these casts have a typical RTE cell appearance (see Web Color Image 9–12). Casts containing a mixture of cells are referred to as “mixed cell casts.”

6. Granular casts may be degenerated cellular casts or they may represent protein aggregation on the Tamm-Horsfall cast matrix. They are classified as either finely granular (see Web Color Image 9–13) or coarsely granular (see Web Color Image 9–14) based on the appearance of the inclusions. Granular casts are always associated with renal disease, either glomerular or tubulointerstitial. Waxy casts are the final degenerative stage of finely granular casts. These casts are smooth with blunt ends and cracks along...
Figure 9–2 Types of urinary casts: their appearance and clinical significance.

<table>
<thead>
<tr>
<th>Type</th>
<th>Appearance</th>
<th>Clinical Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hyaline</td>
<td>Colorless, homogeneous protein matrix with rounded ends; various sizes and shapes</td>
<td>Strenuous exercise, stress Inflammation of the urogenital tract Often found with other casts</td>
</tr>
<tr>
<td>Red blood cell</td>
<td>Protein matrix filled with red blood cells; often many free red cells in microscopic field</td>
<td>Glomerulonephritis Acute interstitial nephritis Strenuous exercise</td>
</tr>
<tr>
<td>White blood cell</td>
<td>Protein matrix filled with white blood cells</td>
<td>Pyelonephritis Kidney infection (accompanied by bacteria, protein, red blood cells) Glomerulonephritis (accompanied by red blood cell casts)</td>
</tr>
<tr>
<td>Epithelial cell</td>
<td>Protein matrix filled with renal epithelial cells</td>
<td>Renal tubular damage</td>
</tr>
<tr>
<td>Fine or coarsely granular</td>
<td>Protein matrix filled with degenerating cells, amorphous crystals, or bacteria; colorless to yellow; many shapes and sizes</td>
<td>Urine flow stasis Urogenital tract infections Strenuous exercise, stress</td>
</tr>
<tr>
<td>Fatty</td>
<td>Protein matrix containing oval fat bodies</td>
<td>Nephrotic syndrome Renal tubular cell death Severe crush injury with disruption of body fat accompanied by significant proteinuria</td>
</tr>
<tr>
<td>Waxy or broad</td>
<td>Homogeneous with waxy, thick appearance, often with blunt, uneven, brittle-looking edges</td>
<td>Tubular obstruction or disease with extreme urine flow stasis Severe nephron damage Nephrotic syndrome Poor prognosis with large numbers present</td>
</tr>
<tr>
<td>Pigmented</td>
<td>Hyaline matrix with coloration due to pigment incorporation</td>
<td>Incorporated bilirubin (golden-brown) Hemoglobin or myoglobin (yellow to red-brown)</td>
</tr>
<tr>
<td>Mixed</td>
<td>Two or more cell types enmeshed in a protein matrix; part granular and part waxy Any combination is possible</td>
<td>Two cell types in filtrate during cast formation; initial cast formation in one tubule, followed by stasis in another wider lumen and further cast formation</td>
</tr>
</tbody>
</table>

1. Waxy casts in urine are a sign of renal failure or severe nephron damage.

7. Other casts include fatty casts, pigmented casts, bacterial, fibrin, and crystal casts depending on the inclusions within the protein matrix.

D. Crystals are commonly found in urine sediment but are rarely clinically significant. Precipitated crystals appear in various forms or as amorphous material. Crystal identification is based on microscopic appearance and urine pH. Normal crystals can be found in acid,
Table 9–5 Summary of Normal Urinary Crystals

<table>
<thead>
<tr>
<th>Crystal</th>
<th>pH</th>
<th>Color</th>
<th>Solubility</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uric acid</td>
<td>Acid</td>
<td>Yellow-brown</td>
<td>Alkali or heat</td>
</tr>
<tr>
<td>Amorphous urates</td>
<td>Acid</td>
<td>Brick dust or yellow-brown</td>
<td>Alkali and heat</td>
</tr>
<tr>
<td>Calcium oxalate</td>
<td>Acid, neutral, alkaline</td>
<td>Colorless (envelopes)</td>
<td>Dilute HCl</td>
</tr>
<tr>
<td>Amorphous phosphate</td>
<td>Alkaline, neutral</td>
<td>White-colorless</td>
<td>Dilute acetic acid</td>
</tr>
<tr>
<td>Calcium phosphate</td>
<td>Alkaline, neutral</td>
<td>Colorless</td>
<td>Dilute acetic acid</td>
</tr>
<tr>
<td>Triple phosphate</td>
<td>Alkaline</td>
<td>Colorless (coffin lids)</td>
<td>Dilute acetic acid</td>
</tr>
<tr>
<td>Ammonium biurate</td>
<td>Alkaline</td>
<td>Yellow-brown (thorny apples)</td>
<td>Acetic acid with heat</td>
</tr>
<tr>
<td>Calcium carbonate</td>
<td>Alkaline</td>
<td>Colorless (dumbbells)</td>
<td>Gas from acetic acid</td>
</tr>
</tbody>
</table>

Normal crystals found in acidic urine are urates (i.e., uric acid, amorphous urates) and calcium oxalate (Table 9–5 and Figure 9–3). Microscopically, all urates appear yellow to reddish-brown.

1. **Uric acid crystals** are yellow to red to orange in color and appear in many shapes, including four-sided and flat; rhombic plates or prisms; ovals with pointed ends; rosettes; wedges; and needles. They are best identified with polarized light, under which they are multicolored (see Web Color Images 9–15 and 9–16).

2. **Amorphous urates** are yellow-brown granules, often found in clumps that may obscure other elements present in the urine sediment. When present in large amounts, they make the urine specimen appear pink-orange or reddish-brown and turbid. They are easily solubilized by heating the urine specimen (see Web Color Image 9–17).

3. **Calcium oxalate crystals** can be seen in acidic or neutral urine. On rare occasions, they are found in alkaline urine. These crystals usually appear under the microscope as small, colorless octahedrals that resemble envelopes or with a cross on their surface. They can also appear in a dumbbell shape (see Web Color Image 9–18).

4. **Normal crystals found in alkaline urine** (Table 9–5 and Figure 9–3) are predominantly phosphates, which include triple phosphate, amorphous phosphates, and calcium phosphate. Other crystals found in alkaline urine are ammonium biurate and calcium carbonate.

   a. **Triple phosphate crystals** have a distinct colorless, three- to six-sided prism shape with oblique ends and are often called coffin lids (see Web Color Image 9–19).

   b. **Amorphous phosphates** are granular in appearance. When present in large numbers, they give the urine specimen a white turbidity. They can mask other elements.
present in the urine sediment. Dilute acetic acid dissolves some of the crystals, but can also lyse any RBCs that may be present (see Web Color Image 9–20).

e. **Calcium phosphate** (see Web Color Image 9–21) appears as colorless, thin prisms, plates, or needles. These crystals are not frequently seen in urine, but when present can be confused with sulfonamide crystals (see image below), which are abnormal. The two are distinguished by adding dilute acetic acid to the urine sediment. Calcium phosphate is soluble; sulfonamides are insoluble.

d. **Ammonium biturate crystals** are yellow-brown spheres with irregular projections or “thorns” and are referred to as thorny apples. They are often seen in old specimens (see Web Color Image 9–22).

e. **Calcium carbonate crystals** are small, colorless dumbbells or spheres. They often appear in clumps and can be confused with amorphous phosphates. They are distinguished by the formation of carbon dioxide gas after the addition of acetic acid (see Web Color Image 9–23).

3. **Abnormal crystals** are found in acidic or neutral urine and have characteristic shapes (Figure 9–4; Table 9–6). No abnormal crystals are found in alkaline urine. Abnormal crystals found in urine are cystine, cholesterol, leucine, tyrosine, bilirubin, sulfonamides, ampicillin, and radiographic dyes. It is important to check the drug therapy of individuals when unusual crystals are found in their urine specimens.

a. **Cystine crystals** are colorless hexagonal plates that precipitate in acidic urine. They result from an inherited metabolic defect that prevents the reabsorption of cystine by the proximal convoluted tubule (see Web Color Image 9–24).

b. **Cholesterol crystals** in acidic urine resemble rectangular plates with a notch in one or more corners. These crystals are seen in urine from individuals with nephrotic

![Table 9-6 Summary of Abnormal Urinary Crystals](image-url)
syndrome or if the lymphatic system has been damaged. They are seen when urine specimens have been refrigerated and in urine specimens with elevated protein (see Web Color Image 9–25).

c. **Leucine** appears as yellow-brown oily looking spheres that contain concentric circles with radial striations (see Web Color Image 9–26). These crystals are observed in urine from individuals with liver failure.

d. **Tyrosine crystals** look like sheaths of fine needles. They are rare, but can occur in individuals who have severe liver disease. They are found in acidic or neutral urine (see Web Color Image 9–27).

e. **Bilirubin** can precipitate in acidic urine as yellowish spheres with spicules. They appear in urine from individuals with liver disease and are composed of the conjugated form of bilirubin (see Web Color Image 9–28).

f. **Sulfonamides** most often look like colorless or yellow-brown bundles of wheat with central bindings of wheat that have been refrigerated. Depending on the form of the sulfonamide drug the individual is taking, these crystals can appear as rosettes, arrowheads, needles, petals, or round forms with striations. They usually appear when individuals are not adequately hydrated (see Web Color Image 9–29).

g. **Ampicillin crystals** appear as long, fine colorless needles or form coarse sheaves after refrigeration.

h. **Radiographic dyes** (contrast media) may have many colorless forms and can be confused with uric acid crystals or calcium carbonate crystals. When radiographic dyes are present in a urine specimen, the specific gravity is very high (>1.040) (Web Color Image 9–30).

E. **Microorganisms, artifacts, and miscellaneous**

1. **Bacteria** may or may not be significant, depending on the method of specimen collection and how soon after collection the specimen is examined. If WBCs are also present in the sediment with bacteria, an infection may exist. Bacteria will appear as rods, cocci, cocci in chains, or all of these and are typically reported as few, moderate or many under high power (Web Color Image 9–31).

2. **Yeast**s are usually found in the urine of individuals who have diabetes, but may also have gained access to the urine from places they usually reside (e.g., skin, vaginal tract) as the urine is voided. Airborne yeasts may also contaminate a urine specimen if it is left uncovered. Unless they are budding, yeasts can be confused with RBCs. To differentiate RBCs and yeast, it is best to add a drop of dilute acetic acid to the urine sediment and re-examine it. RBCs lyse; yeast cells remain intact. Occasionally, mycelial forms of *Candida* are seen (see Web Color Image 9–32).

3. The parasite *Trichomonas vaginalis* (8–20 μm) is seen in urine specimens as the result of vaginal contamination. Small species can be confused with WBCs, but the parasite has a characteristic undulating flagella.

4. Ova of the parasite *Schistosoma haematobium* are shed directly into urine. These are large (30 × 80 μm) ovals with a lateral spine (Chapter 9).

5. Occasionally, amoebae can find their way to the bladder through the lymphatics. *Entamoeba histolytica* (cyst 10–20 μm) is usually accompanied by erythrocytes and leukocytes (Chapter 9).

6. *Enterobius vermicularis* (pinworm) eggs or ova (30 × 50 μm) can contaminate urine when the female migrates to the perianal fold to lay its eggs. Other intestinal parasites can be seen in urine that has been contaminated with feces (Chapter 9).

7. Artifacts seen in urine include muscle fibers and vegetable cells seen with fecal contamination, hair, cotton fibers (see Web Color Image 9–33) from diapers or other cloth materials, starch granules from surgical gloves (see Web Color Image 9–34), and oil droplets from lubricants used as catheter lubricants or vaginal creams. If glass particles are on a slide or coverslip used to examine urine sediment, these may appear as bright, refractile, and irregularly shaped objects (see Web Color Image 9–35).

8. **Mucus**, a protein material produced by glands and epithelial cells in the urogenital tract, is commonly observed in urine specimens, but has no clinical significance.
CHAPTER 9  Urinalysis and Body Fluid Analysis

Microscopically, mucus appears as threadlike structures with low refractive indexes, which requires subdued light for observation. The irregular appearance of mucus helps to differentiate it from hyaline casts (see Web Color Image 9–36).

9. Spermatozoa may be found in a female urine specimen after sexual intercourse and are considered vaginal contaminants. Spermatozoa may be found in the urine of men after recent sexual activity, ejaculation or nocturnal emission. Disease of the genitourinary tract must also be considered if sperm are found in a male’s specimen (see Web Color Image 9–37).

10. Unstained hemosiderin granules appear as coarse yellow-brown granules and are the result of ferritin degradation following a severe hemolytic episode. Hemosiderin can be confused with amorphous crystals. The Prussian blue stain is used to identify hemosiderin granules.

VI. AUTOMATION IN THE URINALYSIS LABORATORY

A. Reflectance photometry
1. Automated reagent strip readers measure the light reflected from the reagent strip color pads and compare the amount of reflected light with a known standard. The concentration of a specific substance and concentration units are displayed on the reader’s display.
2. Principle of reaction: light reflected from the colored reagent pads decreases in a direct proportion to the intensity of the color produced by the reaction with the specific substance in the urine sample (e.g., the darker the color, the less light reflected).

B. Automated urinalysis and microscopy
1. Automated high-throughput urine analyzers utilize laser-based flow cytometry, impedance, and light scatter to identify specific sediment elements in a urine sample; harmonic oscillation is used to assess specific gravity and reflectance photometry reads the reagent strips. Hydrodynamic focusing is another method used to identify specific sediments.
2. Principles of reactions
   a. Harmonic oscillation is a method based upon densitometry in which a sound wave of a specific frequency changes in proportion to the density of the urine sample. The change in wave frequency is measured by a microprocessor and translates the reading to specific gravity.
   b. Laser-based flow cytometry measures sediment conductivity and light scatter. Conductivity is based upon the impedance (the amount of resistance that occurs when a sediment passes through an electrical field) of sediments and counts the numbers of pulses (sediments). The size of the pulse indicates the size of the sediment. Light scattering characteristics of the sediments are determined by their movement through the laser light beam. Identification depends on how the light is scattered by the sediment.
   c. Hydrodynamic focusing involves the movement of single urine sediments past the optics of a microscope to allow sediments to flow in several planes plane past the microscope objective. A flow cell also measures sediment conductivity, size, and light scattering traits. Questionable findings are viewed on a monitor for operator identification and confirmation.

VII. METABOLIC PRODUCTS IN THE URINE (Figure 9–5)

A. Homogentisic acid, a product formed in the metabolic pathway of tyrosine, is excreted in the urine of individuals who lack a specific enzyme and develop alkaptonuria. Two screening tests are used to detect homogentisic acid: the ferric chloride test and the silver nitrate test.
1. A transient, dark blue color is seen as two drops of 10% ferric chloride are added to 2 mL urine.
2. A black color develops after several drops of 10% ammonium hydroxide are added to 0.5 mL urine containing 4 mL of 3% silver nitrate.

B. A second metabolic pathway for tyrosine is responsible for the production of melanin. When melanin and its precursors are present in urine, the urine turns dark, even black, with exposure to light or air. To distinguish melanin from homogentisic acid, two tests can be performed.

1. In the ferric chloride tube test, a gray or black precipitate will form with melanin.
2. A red color is produced with melanin and sodium nitroprusside.

C. Phenylketonuria (PKU) is a well-known aminoaciduria. It occurs in approximately 1 in every 15,000 births, and most states require that newborn infants be tested for this disease. If undetected, this condition results in severe mental retardation. Increased amounts of phenylalanine metabolites in urine give it a characteristic mousy odor.

1. PKU is caused by the failure to inherit the gene that produces the enzyme phenylalanine hydroxylase. The disease is usually first detected in blood because urinary accumulation of phenylpyruvic acid takes 2 to 6 weeks. A newborn must have adequate ingestion of dietary phenylalanine, which is a major constituent of milk, prior to blood collection. Once detected, dietary restrictions that eliminate phenylalanine are necessary. Phenylketonuria is diagnosed by measuring plasma amino acids that indicate elevated plasma phenylalanine and phenylalanine/tyrosine ratio.

2. The best known screening test for PKU is the bacterial inhibition test developed by Guthrie.
   a. Blood is collected on filter paper disks typically using a heelstick procedure.
   b. The blood-impregnated disks are placed on culture media streaked with *Bacillus subtilis* bacteria.
   c. The Guthrie test is sensitive to serum phenylalanine concentrations >4 mg/dL. If increased amounts of phenylalanine are present in the blood, this will counteract the action of an inhibitor present in the media, and *Bacillus subtilis* will grow around the disk.
3. Phenylpyruvic acid can be detected in urine with the ferric chloride reaction. Phenistix reagent strips are available for the detection of PKU. When dipped into urine containing phenylpyruvic acid, a permanent blue-gray to green-gray color is produced.

D. Other disorders of tyrosine metabolism can result from inherited or metabolic defects. If tyrosine derived from the diet or from the metabolism of phenylalanine is not metabolized, it accumulates in the serum up to 100 times normal, producing tyrosinemia and overflow into the urine (tyrosyluria).
1. Cirrhosis of the liver, renal dysfunction, and rickets are the principal clinical findings in hereditary tyrosinemia, which is rare. More often, transient tyrosinemia, and thus tyrosyluria, occur in low-birth-weight infants and must be distinguished from PKU.
2. Precipitated tyrosine crystals may be seen in urine sediment. A screening test to detect tyrosine in urine uses nitrosonaphthol, which forms red complexes with tyrosine and tyramine, but is nonspecific.
3. Chromatography should be used to confirm the presence of increased levels of tyrosine because normal urine contains some tyrosine.

E. Maple syrup urine disease (MSUD) is one of a group of disorders associated with abnormal branched-chain amino acids.
1. Failure to inherit the gene for the enzyme necessary to produce oxidative decarboxylation of the keto acids in the metabolic pathways of leucine, isoleucine, and valine results in their accumulation in the blood and urine. These excess keto acids give urine a characteristic maple syrup odor.
2. The most commonly used screening test for keto acids is the 2,4-dinitrophenylhydrazine (DNPH) reaction. Addition of DNPH to urine containing keto acids produces a yellow turbidity or precipitate.

F. Miscellaneous tests for metabolic products
1. Dysfunction in the metabolism of tryptophan can result in an increase of indican or 5-hydroxyindoleacetic acid (5-HIAA) in the urine.
   a. In certain intestinal disorders, including obstruction, the presence of abnormal bacteria, malabsorption syndromes, or a rare inherited disorder (Hartnup disease), increased amounts of tryptophan are converted to indole in the intestine.
   b. The excess indole is then reabsorbed into the blood and converted to indican by the liver and excreted into the urine.
   c. Indican in urine, when exposed to air, is oxidized to indigo blue.
   d. Urinary indican is detected by an acidic ferric chloride solution, which reacts with indican to form a deep-blue or purple color.
2. In another metabolic pathway, tryptophan is converted to serotonin in the argentaffin cells of the intestine.
   a. Malignant tumors of the argentaffin cells produce excess amounts of serotonin, which results in elevated levels of the urinary degradation product 5-HIAA.
   b. 5-HIAA can be detected in urine with the addition of 1-nitroso-2-naphthol, which produces a purple to black color, depending on the concentration of 5-HIAA.
3. Cystinuria is characterized by defective tubular reabsorption of cystine and the amino acids arginine, lysine, and ornithine after glomerular filtration.
   a. The demonstration of multiple amino acids not being reabsorbed rules out the possibility of an error in metabolism, although the condition is inherited. Approximately 65% of individuals who have cystinuria tend to form calculi.
   b. A fresh, first morning urine specimen should be examined for cystine crystals.
   c. A chemical screening test for urinary cystine uses the cyanide-nitroprusside test. Sodium cyanide reduces cystine, and the free thiols then react with nitroprusside to produce a red-purple color.
4. Homocystinuria is caused by deficiency of the liver enzyme cystathionine β-synthase. Homocysteine is rapidly oxidized to homocystine, which accumulates and is excreted in the urine. Children afflicted with this disease may have seizures and thromboses, and they may become mentally retarded.
A fresh urine specimen should be tested for homocystine, because it is labile.

The cyanide-nitroprusside reaction is positive.

G. Urine calcium

1. Elevated urinary calcium may be seen in individuals who have renal calculi, hyperparathyroidism, osteoporosis, or multiple myeloma. Occasionally, individuals who take large amounts of calcium supplement might produce high urine calcium. Sulkowitch’s test is a quick qualitative test for increased levels of urinary calcium. Sulkowitch’s reagent consists of oxalic acid, ammonium oxalate, and glacial acetic acid. When reacted with urinary calcium, calcium oxalate precipitates, producing turbidity that is graded on a scale from 0 to 4.

2. If large amounts of clumped calcium oxalate or calcium carbonate crystals are noted in a freshly voided urine sample, it may be predictive of the presence of renal calculi.

H. Urine porphyrins

1. Porphyrins are intermediate compounds in the production of heme.

2. Porphyrrias are a group of disorders resulting from defects in the heme synthesis pathway.

   a. Inherited enzyme deficiencies and lead poisoning interrupt the heme synthesis pathway and produce porphyrins, which result from the spontaneous and irreversible oxidation of their respective porphyrinogens. These porphyrins cannot re-enter the heme synthesis pathway and are excreted in urine and stool.

   b. Porphyrrias are rare disorders. The most common type in North America is porphyria cutanea tarda.

3. The principle circulating porphyrins include uroporphyrin, coproporphyrin, and protoporphyrin.

4. Porphyrin precursors commonly found in urine are porphobilinogen and aminolevulinic acid (ALA).

5. In their oxidative forms, uroporphyrin, coproporphyrin, and protoporphyrin are dark red or purple and fluorescent. The oxidative form of porphobilinogen—porphobilin—is dark red. Thus, urine containing porphobilinogen and porphobilin may appear dark red, often referred to as “port wine” color.

6. If test results are negative for blood in red-colored urine and the individual is not taking medication that could color the urine, the specimen should be tested for porphyrinuria.

7. There are two screening tests for porphyrinuria: the Ehrlich’s reaction and fluorescence under ultraviolet light from a Wood’s lamp (see 10 below). In Ehrlich’s reaction, urobilinogen and porphobilinogen react with p-dimethylaminoazobenzaldehyde (Ehrlich’s reagent) to produce a cherry-red color.

8. To detect ALA, acetylacetone is added to a urine specimen before testing to convert ALA to porphobilinogen. Addition of Ehrlich’s reagent produces a cherry-red color in a specimen that yields positive test results. Increased urinary ALA is a common screening test for lead poisoning.

9. If a cherry-red color is produced by a urine specimen after the addition of Ehrlich’s reagent, the Watson-Schwartz test is performed to differentiate urobilinogen and porphobilinogen based on solubility differences.

   a. Chloroform is added to the tube to extract urobilinogen, and porphobilinogen remains in the aqueous phase. The tube is shaken vigorously, and the phases are allowed to separate.

   b. If the red color resides only in the chloroform layer, increased amounts of urobilinogen are present. If the aqueous layer is red, porphobilinogen or other Ehrlich-reactive substances are present.

10. Fluorescence screening detects the presence of urobilinogen, coproporphyrin, and protoporphyrin. These porphyrins must be extracted into a mixture of glacial acetic acid and ethyl acetate. The solvent layer is then examined with a Wood’s lamp. If the test is positive, the solvent layer fluoresces as pink, violet, or red, depending on the concentration of porphyrins present.
11. The Hoesch screening test for porphobilinogen is a rapid screen for urinary porphobilinogen. Two drops of urine are added to the Hoesch reagent, which is Ehrlich’s reagent plus HCl. The uppermost part of the solution turns red in the presence of porphobilinogen.

VIII. DISEASES OF THE KIDNEY

A. Glomerular diseases

1. Four distinct morphologic changes of the glomeruli are recognized in glomerular disease: cellular proliferation, leukocyte infiltration, glomerular basement-membrane thickening, and hyalinization with sclerosis (hardening). Hyalinization is the deposition of an albuminoid mass in tissues that typically appears semi-transparent in H&E stains.
   a. Cellular proliferation includes increased numbers of epithelial cells, mesangial cells, and endothelial cells, which compose the capillary endothelium and accumulate in the glomerular tuft.
   b. Leukocyte infiltration results from local chemotactic response or cellular proliferation. Neutrophils and macrophages can readily infiltrate glomeruli in response to chemotactic factors.
   c. Glomerular basement-membrane thickening usually results from deposition of precipitated proteins (e.g., immune complexes, fibrin).
   d. Hyalinization of glomeruli results from the accumulation of a homogeneous, eosinophilic extracellular material in the glomeruli, which causes the glomeruli to lose their structural detail and become sclerotic.

2. Immunologic disorders are the primary cause of glomerular injury. Circulating immune complexes created in response to either endogenous (e.g., cellular) or exogenous antigens (e.g., microorganisms) become trapped in the glomeruli. A second immune response involves auto-antibodies that react directly with antigens on the glomerular tissue. These may be cross-reacting antibodies formed against exogenous antigens. Glomerular injury results from the toxic substances (e.g., proteases, oxygen-derived free radicals, and arachidonic acid metabolites) produced by the complements, neutrophils, monocytes, platelets, and other factors at the site of antibody deposition.

3. Glomerulonephritis is an inflammatory process that affects the glomerulus and includes some or all of these clinical findings: hematuria, proteinuria, oliguria, azotemia, edema, and hypertension.
   a. Acute glomerulonephritis refers to a disease characterized by the rapid onset of symptoms (e.g., fever, malaise, nausea, oliguria) caused by an inflammatory process in the glomerulus. Blood, protein, and casts, particularly RBC casts, are detected in urine.
      (1) Poststreptococcal glomerulonephritis is most frequently seen in children or young adults following a respiratory tract infection caused by group A streptococci (e.g., *Streptococcus pyogenes*) in which treatment either did not occur, or it failed. As the bacteria proliferate and the body forms antibodies to them, opsonized bacteria form immune complexes that deposit on the glomerular membrane.
      (2) Approximately 95% of children and 60% of adults recover spontaneously or with minimal therapy. The remainder recovers more slowly or develops chronic glomerulonephritis.
   b. Crescentic (rapidly progressive) glomerulonephritis is a more serious type of glomerulonephritis that often terminates in renal failure. Urinalysis findings include hematuria, proteinuria, oliguria; RBCs; and hyaline, granular, RBC, and WBC casts.
      (1) It is characterized by cellular proliferation of epithelial cells inside Bowman’s capsule that form “crescents,” which cause pressure changes in the glomerular
tuft and can occlude the entrance to the proximal tubule. Fibrin deposition and leukocyte infiltration are present.
(2) Fibrin degradation products and IgA complexes eventually appear in urine, while extremely elevated serum protein occurs as the disease progresses.

c. Membranous glomerulonephritis is characterized by thickening of the glomerular capillary basement membrane, which is usually caused by immune complex deposition. The clinical course of this disease is variable, but may progress to a nephrotic syndrome. Urinalysis findings include hematuria and proteinuria.

d. Mesangiocapillary (membranoproliferative) glomerulonephritis is characterized by cellular proliferation and leukocyte infiltration that leads to thickening of the glomerular basement membrane. Immune complex deposition and complement activation over many years leads to chronic renal failure. Laboratory findings may include hypocomplementemia, hematuria, and proteinuria.

e. In focal glomerulonephritis, only a certain number of glomeruli are sclerotic. Characteristic pathology findings include immune complex deposition with hematuria and proteinuria.

f. Chronic glomerulonephritis results from continual or permanent damage to the glomerulus associated with irreversible loss of renal tissue and chronic renal failure. Clinical signs include edema, hypertension, anemia, metabolic acidosis, and oliguria that progresses to anuria. Urinalysis reveals hematuria, proteinuria, azotemia, and many types of casts including broad or waxy.

B. Tubular and interstitial diseases

1. Acute tubular necrosis results from destruction of renal tubular epithelial cells, which is caused by decreased perfusion of the kidneys and ischemia (decreased blood flow) or certain agents (e.g., hemoglobin, myoglobin, drugs) or toxins (e.g., heavy metals, poisons, organic solvents) that produce ischemia.

a. The resulting oliguria leads to acute renal failure.

b. Urinalysis findings include proteinuria, hematuria, pyuria, and many types of casts, depending on the phase of the disease.

2. Tubular dysfunction can be a primary renal disease or secondary to some other disease process.

a. Fanconi's syndrome is any condition that is characterized by a generalized proximal tubular dysfunction. As a result, the reabsorption of water, glucose, phosphorus, potassium, calcium, and amino acids is decreased, and these substances are excreted into the urine.

b. Cystinosis is an inherited trait that results in the intracelluar deposition of cystine. Every cell in the body is affected, including the proximal and distal tubular cells. The kidney can no longer concentrate or acidify urine. The extensive renal cell damage eventually requires dialysis or transplantation.

3. Tubulointerstitial disease results from other diseases that affect the renal tubules and eventually involve the interstitium. Causes of tubulointerstitial diseases are infections, toxins (e.g., drugs, heavy metals), vascular or metabolic diseases, irradiation, neoplasms and organ transplant rejection.

4. Urethritis is microbial infection of the urethra. Cystitis is microbial infection of the bladder. Pyelitis is infection of the renal pelvis. Pyelonephritis is infection of the renal pelvis and the interstitium.

5. UTIs are more common in women than men, and 85% of UTIs are caused by gram-negative enteric bacteria. The most common pathogen is *Escherichia coli*. UTIs cause
pain or burning on urination with urinary frequency and occasional back pain. Urinalysis findings include: pyuria (i.e., presence of WBCs) and bacteria with mild hematuria and proteinuria possible as well. Microscopic examination of an upper UTI might reveal WBC and granular casts; these are not seen in lower UTIs.

6. **Acute pyelonephritis** is an acute infection of the renal tubules, interstitium, and renal pelvis.
   a. There are two possible mechanisms leading to acute pyelonephritis:
      (1) Bacteria can ascend the ureters from a lower UTI; or
      (2) Bacteremia can occur with bacteria localizing in the kidney.
   b. Bacteria multiply in the kidney, which causes inflammation that spreads to the tubules. Large numbers of neutrophil enzymes and bacterial toxins cause necrosis of the tubules.
   c. Urinalysis reveals pyuria with WBCs often in clumps, large numbers of bacteria, leukocyte casts (and possibly granular, renal cell, or broad casts), proteinuria, and hematuria.

7. Yeast infections of the urinary tract usually result from inoculation of these organisms into the urinary tract by catheters or from the bloodstream, if it is infected with yeast.

8. **Chronic pyelonephritis** is a chronic inflammation of the tubulointerstitial tissues and results in permanent scarring.
   a. Urinalysis findings include pyuria and proteinuria. Bacteria and casts may or may not be present.
   b. Polyuria and nocturia develop as tubular function is lost, which results in low specific gravity of urine.

9. **Acute interstitial nephritis** is a condition of renal injury that results from various drugs and toxins. Urinalysis results include hematuria, proteinuria, WBCs (particularly eosinophils) and WBC casts with no bacteria.
   a. Drugs, such as penicillin and sulfonamides, can produce an immediate allergic reaction in the renal interstitium, which is caused by the binding of the allergen to the interstitial protein.
   b. Heavy metals and aminoglycosides can cause direct damage to tubules.

10. **Acute renal failure (ARF)** results from tubular necrosis with marked loss of renal function. Prerenal causes of ARF (systemic disease such as cardiac or pulmonary failure) that produce decreased blood flow to the kidneys, renal causes (within the kidneys such as glomerular disease), and postrenal causes (obstruction of the renal system such as tumors or calculi) all can lead to ARF. Typical symptoms produced by ARF include:
    a. Decreased glomerular filtration rate and oliguria
    b. Lack of renal concentrating ability
    c. Increased serum BUN and creatinine levels
    d. Microscopic findings of casts or renal tubular epithelial cells in prerenal disease, RBCs and cellular casts in renal causes, and tumor cells or large numbers of clumped calcium oxalate crystals in obstructive postrenal disease.

11. **Chronic renal failure** results from a progressive chronic loss of renal function and can be caused by progression from the original renal disorder. Urinalysis findings consist of isoosmolar at 1.010 (caused by the loss of kidney concentrating ability), significant proteinuria, hematuria, and a telescoped urine sediment (includes all types of casts: granular, broad and waxy). Serum urea nitrogen and creatinine rise gradually.

C. **Other renal-related diseases**

1. **Systemic diseases** producing glomerular damage include systemic lupus erythematosus (SLE), amyloidosis, and diabetes mellitus.
   a. In individuals who have SLE, immune complexes (e.g., DNA and anti-DNA) are deposited in the glomerular membrane.
      (1) Complement is activated, inflammatory cells are called to the area, and the cellular proteases destroy the surrounding tissue.
Disease progression may lead to recurrent hematuria, acute nephritis, or nephrotic syndrome.

Chronic renal failure is a leading cause of death in SLE individuals who have renal involvement.

**b. Amyloidosis** is characterized by deposition of amyloid between cells in any organ or tissue of the body. Amyloid is a substance composed of 90% fibril protein and 10% glycoprotein.

1. The deposition of amyloid within the glomeruli eventually destroys them.
2. Clinical findings include proteinuria and nephrotic syndrome. Eventually, renal failure and uremia occur as more and more glomeruli are destroyed.

**c. Diabetes mellitus** can result in renal disease. Renal capillaries, as are capillaries in the retina and periphery, are markedly affected if the diabetes is uncontrolled.

1. Prolonged glycosemia is toxic to the kidney, particularly the glomerulus, and causes thickening of the basement membrane.
2. These individuals have an excessive thirst (polydipsia), which leads to high volume urine with a high specific gravity because of the glucose it contains.
3. Chronic renal failure eventually develops with persistent proteinuria.

**2. Nephrotic syndrome** is characterized by pronounced proteinuria (i.e., ≥3.5 g/day). Systemic diseases that can lead to nephrotic syndrome include SLE, amyloidosis, systemic shock, untreated infection, malignancies, and uncontrolled diabetes mellitus. Nephrototoxic agents such as drugs and poisons can also lead to nephrotic syndrome.

**a. Many plasma proteins, particularly albumin, are excreted into urine because of increased permeability of the glomeruli.**

**b. Other clinical findings include hypoproteinemia (particularly albumin), hyperlipidemia, lipuria, and edema.**

**c. Decreased plasma immunoglobulins and other low-molecular-weight molecules (e.g., complement components, coagulation factors) make these individuals more susceptible to infections and thrombosis.**

**d. Urinalysis findings include marked proteinuria; lipuria with oval fat bodies; hematuria; and fatty, waxy, and renal tubular epithelial cell casts.**

**3. Renal calculi** are solid aggregates of mineral salts that form within various glands. Calculi of the urinary tract are called kidney stones. They can be found in the renal calyces, pelvic, ureters, or bladder.

**a. Calculi are usually composed of a mixture of elements. Most renal calculi contain calcium and oxalate. Other frequently seen components are phosphates, uric acid, and cystine.**

**b. Causes of calculi formation include underlying metabolic or endocrine disorders, infections, or a fixed urinary pH. When the urine pH is optimal, and the urine becomes supersaturated with a particular chemical component, renal calculi tend to form.**

**c. Classification of renal calculi**

1. Urinary calculi are classified by location and chemical nature. In the United States, most urinary calculi are found in kidneys or ureters. Multiple calculi and bladder calculi are less common.
2. Calcium calculi are the most common in the United States.
3. Techniques for analyzing calculi include chemical and physical analysis, polarizing microscopy, and others.

**D. Causes of various calculi compositions**

1. Calculi of calcium composition are caused by the following diseases or physiologic conditions:
   a. Idiopathic hypercalciuria
   b. Primary hyperparathyroidism
   c. Bone disease
   d. Excessive milk, alkali, or vitamin D intake
   e. Renal tubular acidosis
   f. Sarcoidosis
   g. Berylliosis
2. Calculi of calcium oxalate composition are caused by the following diseases or physiologic conditions:
   a. Oxaluria
   b. Incomplete catabolism of carbohydrates
   c. Isohydria at pH 5.5 to 6.0
   d. Excessive glycogen breakdown

3. Calculi of calcium-phosphate composition are caused by the following diseases or physiologic conditions:
   a. Same conditions as for calcium oxalate
   b. Alkaline infection (urea-splitting)
   c. Persistently alkaline urine

4. Calculi of magnesium ammonium phosphate hexahydrate composition are caused by alkaline infection with urea-splitting bacteria.

5. Calculi composed of uric acid and urate are caused by the following diseases or physiologic conditions:
   a. Gout
   b. Polycythemia
   c. Leukemia
   d. Lymphoma
   e. Liver disease
   f. Acid isohydria
   g. Theophylline and thiazide therapy
   h. Conditions associated with rapid protein catabolism

6. Calculi of cystine composition are caused by the following diseases or physiologic conditions:
   a. Transient acute phases of chronic renal diseases
   b. Heavy metal nephrotoxicity
   c. Aminoaciduria
   d. Renal tubular acidosis syndromes

E. Values of pH can be associated with calculi formation.
   1. Urine consistently acid with a pH < 5.5 favors the formation of uric acid, cystine, or xanthine calculi.
   2. Urine consistently acid with a pH between 5.5 and 6.0 favors the formation of calcium oxalate and apatite calculi.
   3. Urine consistently alkaline with a pH of > 7.0 favors the formation of magnesium ammonium phosphate or calcium phosphate calculi.

IX. RENAL SYNTHETIC PRODUCTS

A. Antidiuretic hormone, also called vasopressin, is a peptide hormone produced by the hypothalamus of the brain and stored in the posterior pituitary gland. It directly affects the cells of the distal convoluted and collecting tubules of the kidney to control water reabsorption.

   1. Increased serum levels of ADH cause increased water reabsorption, which leads to a decreased amount of concentrated urine. Increased ADH is typically caused by hypothalamic or pituitary tumor or adenoma. This is often referred to as the Syndrome of Inappropriate ADH excretion (SIADH). Individuals exhibit hyponatremia and water retention with low urine volume of high concentration (elevated specific gravity).

   2. Decreased ADH levels cause decreased water reabsorption, which induces polydipsia (i.e., excessive thirst), polyuria and a large volume (diuresis) of very dilute urine with a low specific gravity. This is referred to as diabetes insipidus. Diabetes insipidus is a disease characterized by polyuria and polydipsia resulting from inadequate ADH secretion or inability of the renal tubules to respond to this hormone. Causes of decreased ADH include pituitary or hypothalamic insufficiency; toxic drugs; tumors, and brain disorders caused by trauma or neoplasms. Decreased renin also affects ADH.
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B. Renin is a proteolytic enzyme (some consider it a hormone) formed and stored by juxta-glomerular cells of the kidney and released into the lymph and the renal venous blood. Renin converts angiotensinogen into angiotensin products. Angiotensin II causes vasoconstriction of renal arterioles and reabsorption of sodium, which directly stimulate the synthesis and secretion of aldosterone by the adrenal cortex.

1. Low plasma volume and a low sodium level stimulate renin secretion, resulting in aldosterone release, which causes sodium retention and reabsorption and potassium loss. This results in water retention, which increases extracellular fluid volume and elevated blood pressure.
   a. As systemic pressure increases, renin production decreases. This produces a decrease in angiotensin and aldosterone levels.
   b. Potassium loss stimulates aldosterone secretion and suppresses renin release, whereas increased potassium decreases renin and aldosterone levels.

2. Lesions found in the kidney or its vascular supply can lead to increased renin levels, resulting in increased aldosterone production and subsequent changes in sodium and potassium excretion as well as possible hypertension.

3. Chronic renal failure can result in low, high, or normal renin levels.

4. Increased aldosterone levels can result from adrenal adenoma, low renin production, potassium wastage, sodium retention, nephrosis, cirrhosis, and heart failure.

C. Erythropoietin (EPO) is a glycoprotein hormone synthesized in specialized renal tubular fibroblasts that are sensitive to low oxygen levels. EPO stimulates bone marrow to produce more RBCs and thus increase the oxygen carrying capacity of the blood. It is also thought that EPO increases the efficacy of iron insertion into heme.

D. Dihydroxycholecalciferol hydrolase is an enzyme synthesized in the juxtaglomerular cells of the kidney that is responsible for activating vitamin D3. This enzyme is under the control of parathyroid hormone.

X. URINE PREGNANCY TESTING

A. Molecular characteristics of chorionic gonadotropin (CG)

1. The glycoprotein hormone CG is produced by the syncytiotrophoblast cells of the placenta approximately 10 days after conception. CG stimulates the corpus luteum in the ovary to synthesize progesterone during the first weeks of pregnancy. The molecule consists of two noncovalently bound polypeptide subunits: α and β.
   a. The α subunit of CG is nearly identical to the α subunit of luteinizing hormone (LH) and follicle-stimulating hormone (FSH); therefore, this subunit of CG cross-reacts with LH in certain bioassay systems to give false-positive results.
   b. The β subunit determines the biologic and immunologic specificity of CG. Urine contains predominantly CGβ (core fragment) and to a lesser degree unmodified CG and CGn (nicked). The rate of clearance varies for the different CGs.

2. In the first 8 weeks of pregnancy, the CG concentration in maternal serum rises geometrically. Detectable amounts (∼5 IU/L) are present 8 to 11 days after conception, which is in the third week of pregnancy as measured from the last menstrual period. For women aged 18 to 40, serum CG concentrations of 5 IU/L or greater are consistent with pregnancy. Subsequently CG concentrations start to decline in serum and urine, and by the end of the second trimester a 90% reduction from peak concentration has usually occurred.

3. Pregnancy testing is not a direct test for pregnancy (i.e., detection of a fetus), but rather is a test to detect the presence of CG.

B. Methods for urine pregnancy testing

1. Home test kit assays are the most commonly used pregnancy tests in the United States. Most kits provide a single test and use an immunochromatographic method to measure urine CG. Detection limits vary from 6.3 to 50 IU/L.
2. First-morning urine specimens are preferred for qualitative urine pregnancy tests because they are concentrated and contain abundant CG. Urine applied to the device is absorbed into a nitrocellulose bed. The CG is concentrated into a narrow band as the urine migrates. A labeled anti-CG antibody in the device binds to the migrating CG and passes through a zone having solid phase capture antibody to CG. The appearance of a colored line indicates a positive test. Positive controls are typically integrated into the test system. False positive results occur in 1% of tests because of the presence in urine of interfering substances, such as proteins, drugs, bacteria, erythrocytes, or leukocytes. False negative results have occurred because of low CG concentration. About half of these qualitative tests will be positive on the day after the first missed menstrual period. Qualitative serum assays are available and tend to be more reliable than urine assays because urine CG concentrations vary considerably.

XI. SEROUS BODY FLUIDS

A. Origin, anatomic relationship and functions

1. The cavities of the body that hold abdominal organs, lungs, and the heart are lined by two membranes consisting of mesothelial cells. The membrane that lines the cavity is referred to as the parietal membrane and the membrane that forms a sac around the organs is the visceral membrane. The small amount of fluid between these two membranes is serous fluid. Serous fluid is an ultrafiltrate of plasma and is produced and reabsorbed at a constant rate. This fluid functions as a lubricant between the membranes of the cavities and to allow free movement of the organs.
   a. The peritoneum encloses abdominal organs. The fluid contained between the visceral and parietal membranes is peritoneal fluid.
   b. The pleural cavity encloses the lungs. The fluid contained between the visceral and parietal membranes here is pleural fluid.
   c. The pericardium encloses the heart. The fluid contained between the visceral and parietal membranes in this cavity is pericardial fluid.

2. Any disruption of the production and reabsorption of serous fluid because of an alteration in the hydrostatic and oncotic pressure in the capillaries of the cavities will cause an increase in fluid volume between the two membranes. This abnormal fluid buildup is an effusion. Abnormal accumulation of fluid in any body cavity indicates an abnormality.

3. There are two kinds of effusions, transudates and exudates.
   a. Transudates result from excess filtration of blood serum across a physically intact vascular wall due to disruption of reabsorption. This occurs in systemic diseases that alter the hydrostatic pressure of the capillaries and include congestive heart failure, hepatic cirrhosis or nephrotic syndrome.
   b. Exudates are the active accumulation of fluid within body cavities associated with inflammation of the membranes and vascular wall damage. Exudates, which are closer to serum in chemical composition, are caused by the following conditions:
      (1) Inflammatory disorders,
      (2) Malignancies,
      (3) Infections.

4. Specimen collection of serous fluids is known as paracentesis and refers to the percutaneous puncture of a body cavity for the aspiration and removal of fluid.
   a. Thoracentesis is the collection of pleural fluid.
   b. Peritoneocentesis is the collection of peritoneal fluid.
   c. Pericardiocentesis is the collection of pericardial fluid.

B. Analysis of serous body fluids

1. Chemical examination of serous fluid differentiates between an exudate and a transudate (Table 9–7). These include:
   a. A total protein evaluation and fluid-to-serum protein ratio can help distinguish between the physiologic basis of different fluids. A serous fluid with a protein
Table 9–7 Comparison of Exudates and Transudates Based on Laboratory Profile

<table>
<thead>
<tr>
<th>Laboratory Value</th>
<th>Exudate</th>
<th>Transudate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clarity</td>
<td>Cloudy</td>
<td>Clear</td>
</tr>
<tr>
<td>Color</td>
<td>Yellow-green</td>
<td>Yellow</td>
</tr>
<tr>
<td>Common cell type</td>
<td>Segmented neutrophils</td>
<td>Mononuclear cells</td>
</tr>
<tr>
<td>White blood cell count (μL)</td>
<td>&gt;1,000 (pleural)</td>
<td>&lt;1,000 (pleural)</td>
</tr>
<tr>
<td></td>
<td>&gt;500 (peritoneal)</td>
<td>&lt;300 (peritoneal)</td>
</tr>
<tr>
<td>Clottable fibrinogen</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Glucose</td>
<td>≤ plasma</td>
<td>Equal to plasma</td>
</tr>
<tr>
<td>Total protein</td>
<td>&gt;50% of plasma value</td>
<td>&lt;50% of plasma value</td>
</tr>
<tr>
<td>Fluid-to-plasma total protein ratio</td>
<td>&gt;0.5</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>Lactate dehydrogenase</td>
<td>&gt;60% of plasma value</td>
<td>&lt;60% of plasma value</td>
</tr>
</tbody>
</table>

A value >50% of the serum value is considered an exudate, whereas a fluid protein <50% of the serum value is a transudate. Serum albumin-to-fluid albumin ratios are recommended for assessment of hepatic transudates.

b. A lactate dehydrogenase (LD) test with a fluid-to-serum LD ratio can also be used to confirm the formation of the fluid. For transudates, the ratio of fluid LD to serum LD is <0.6; for exudates the ratio is >0.6.

c. Transudate glucose levels are equivalent to the plasma glucose levels; however, exudate glucose levels are low compared to plasma glucose levels.

d. Amylase determination aids in the diagnosis of pancreatitis, bowel perforation, or metastasis.

e. Triglyceride testing can confirm a chylous effusion; cholesterol analysis is performed to assess a pseudochylous effusion.

f. A pH value for pleural fluids is helpful for identifying effusions with abnormally low pH values.

g. Carcinoembryonic antigen (CEA) determination is useful in evaluating effusions from individuals who have a past or current diagnosis of a CEA-producing tumor.

h. Assessment of spontaneous clotting.

2. Microbiological examination of serous fluids for bacteria or Mycobacteria includes Gram’s stain, bacterial culture, and sensitivity studies. Serological tests include antinuclear antibody and rheumatoid factor analyses to assess immunologic disease.

3. Physical examination of serous body fluids includes examination of appearance, cell count, and differential. See Table 9–7 for details.

a. Transudates are typically clear while exudate fluid is cloudy. Bacteria produce white, turbid fluid and the presence of blood in the fluid can indicate malignancy. Blood in pleural fluid can signify the occurrence of hemothorax; in pericardial fluid it can indicate cardiac puncture; in peritoneal fluid it also signifies trauma. Milky-appearing fluid indicates the presence of chylous material (triglycerides) from the thoracic duct or pseudochylous material (cholesterol) from chronic inflammation.

b. Cells are differentiated using cytospin preparations. Typically, a 50- or 100-cell differential is performed. Cells found in normal serous fluid include the following (see Web Color Image 9–38):

(1) Lymphocytes
(2) Monocytes and macrophages
(3) Mesothelial cells from the parietal and visceral membranes

c. Nonmalignant cells found in disease states include the following:

(1) Neutrophils can be found in an exudate during the early stage of inflammatory diseases (see Web Color Image 9–39).
(2) Eosinophils found in serous fluids are associated with infections, malignancy, myocardial infarction, and hypersensitivity reactions.
(3) RBCs can occur in association with hemorrhage, malignancy, or traumatic puncture.
d. Malignant cells can be found in serous fluids in individuals who have leukemia, lymphoma, or metastatic tumors. Differentiating malignant cells from mesothelial cells in particular is difficult. In cases of mesothelioma, a greatly increased number of reactive mesothelial cells are observed in pleural fluid.

e. A lack of mesothelial cells in a pleural fluid sample indicates tuberculosis and is caused by the exudate fluid coating the pleural membranes.

XII. SYNOVIAL FLUID

A. Origin and function of synovial fluid. Synovial fluid is present within the synovial cavity in free-moving joints with the largest amount located in the knee joint cavity. It is produced as an ultrafiltrate of plasma across the synovial membrane and has a chemical composition similar to plasma. This fluid functions to lubricate the joints and to provide the sole nutrient source for the joint tissue.

B. Synovial Fluid Analysis

1. Indications for synovial fluid analysis include arthritis (degeneration of the articular membranes) and other joint diseases. Joint disorders are classified as noninflammatory, inflammatory, septic, and hemorrhagic. Specimen collection of synovial fluid is referred to as arthrocentesis.

   a. The degree and possible causes of joint inflammation can be assessed with a synovial fluid analysis.

   b. Synovial fluid can also be removed from a joint to provide therapeutic benefit (i.e., to remove excess fluid or blood from an inflamed joint).

2. Routine synovial fluid analysis

   a. Physical examination consists of color, clarity, viscosity, and clot formation observations (Table 9–8).

      (1) A yellow color is normal. Color abnormalities of red or brown are associated with joint trauma, and infections can produce a greenish joint fluid.

      (2) Clarity can be altered by RBCs, WBCs, crystals, bacteria, fibrin, or cellular debris.

      (3) Viscosity is related to the concentration of the mucoprotein hyaluronic acid (necessary for joint lubrication) present in the specimen. Viscosity can be decreased in inflammatory, septic or hemorrhagic conditions.

Table 9–8 Normal Synovial Fluid Parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Typical Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume</td>
<td>Up to 3.5 mL</td>
</tr>
<tr>
<td>Color</td>
<td>Straw to yellow</td>
</tr>
<tr>
<td>Clarity</td>
<td>Clear</td>
</tr>
<tr>
<td>Viscosity</td>
<td>High</td>
</tr>
<tr>
<td>Clot formation</td>
<td>None</td>
</tr>
<tr>
<td>Red blood cell count</td>
<td>&lt; 2,000 μL</td>
</tr>
<tr>
<td>White blood cell count</td>
<td>&lt; 200 μL</td>
</tr>
<tr>
<td>Differential</td>
<td></td>
</tr>
<tr>
<td>Neutrophils</td>
<td>50%–70%</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>20%–40%</td>
</tr>
<tr>
<td>Monocyte/macrophage</td>
<td>5%–15%</td>
</tr>
<tr>
<td>Crystals</td>
<td>None</td>
</tr>
<tr>
<td>Glucose</td>
<td>Equal to plasma</td>
</tr>
<tr>
<td>Urea acid</td>
<td>Equal to plasma</td>
</tr>
<tr>
<td>Total protein</td>
<td>1–3 g/dL</td>
</tr>
<tr>
<td>Lactic acid</td>
<td>Equal to plasma</td>
</tr>
<tr>
<td>Hyaluronic acid</td>
<td>0.3–0.4 g/dL</td>
</tr>
</tbody>
</table>
Viscosity can be estimated by expelling a drop of the synovial fluid from a syringe. Synovial fluid of high viscosity (i.e., normal fluid) forms a "string" of 4 to 6 cm before breaking.

(b) Viscosity is also examined by looking at clot formation, which indicates the presence of fibrinogen when the synovial membrane is damaged or when there is hemorrhage or blood contamination during a traumatic tap. The test is referred to as a Ropes or mucin clot test, and is performed by adding synovial fluid to a 2% to 5% solution of acetic acid. Formation of a solid clot indicates good viscosity.

Microscopic examination of synovial fluid includes a hemacytometer count of RBCs and WBCs (Chapter 3), a cytospin differential to determine the types of WBCs, and a wet preparation examination for crystals (Table 9–8 for normal values). Synoviocytes, which are cells that make up the synovial membrane, are similar to mesothelial cells and are a normal finding in synovial fluid. Other cells, such as LE, Reiter cells, and ragocytes, are indicative of specific syndromes.

(1) An increase in RBCs indicates joint hemorrhage or traumatic tap.

(2) A WBC count >2,000/μL, with a predominance of neutrophils, is associated with bacterial arthritis, acute gouty arthritis, or rheumatoid arthritis (see Web Color Image 9–40). The presence of lymphocytes indicates nonspecific inflammation.

(3) Eosinophils may be present in cases of rheumatic fever or parasitic infestation.

(4) LE cells are neutrophils that contain ingested cellular material and indicate lupus erythematosus; they are rarely seen in synovial fluid. Reiter cells are vacuolated macrophages containing an ingested neutrophil and indicate Reiter’s syndrome (systemic inflammation). Ragocytes are neutrophils containing precipitated immunoglobulins, antinuclear antibodies, and rheumatoid factor and are common in septic and rheumatoid arthritis and gout.

(5) The presence of crystals can be diagnostic of joint disease.

(a) Monosodium urate monohydrate crystals are fine, needle-like crystals associated with gouty arthritis. When examined under polarized light, these crystals appear as thin yellow needle-like structures (see Web Color Image 9–41).

(b) Calcium pyrophosphate dihydrate crystals are rodlike or rhombic crystals associated with pseudogout. When examined under polarized light, these crystals appear as bluish plates and rods.

(c) Cholesterol crystals are flat, plate-like crystals with notched corners. These are seen in conditions of chronic rheumatoid arthritis.

(d) Calcium hydroxyapatite crystals can also be present, but can be seen only with an electron microscope.

Chemical examination of synovial fluid includes only a few diagnostically useful tests (see Table 9–8 for normal values).

(1) A decrease in synovial glucose that exceeds a plasma-fluid difference of >10 mg/dL indicates an inflammatory condition or septic disorder.

(2) Increased protein levels indicate synovium permeability changes and damage to the synovial membrane. Large molecules, such as protein, are not filtered through the synovial membrane.

(3) An increase in fluid uric acid levels helps diagnose gout when fluid crystals are not found in the fluid.

(4) Increased lactate concentration is thought to occur from conditions of increased anaerobic glycolysis in the synovial cavity, which is associated with severe inflammatory conditions, such as septic arthritis. Gonococcal arthritis produces normal-to-low lactate levels.

A microbiologic examination can aid in the differential diagnosis of joint disease.

(1) A Gram stain should be performed on all samples. A culture using enriched medium is performed when Hemophilus or Neisseria infections are suspected.

(2) The majority of infectious agents in septic arthritis are bacterial, but can also include fungal, mycobacterium, or viral infections.
Chapter 9: Urinalysis and Body Fluid Analysis

XIII. SEMINAL FLUID ANALYSIS

A. Seminal fluid physiology

1. Seminal fluid is composed of secretions from the testes, epididymis, seminal vesicles, and prostate gland.
   a. Spermatozoa cells form approximately 5% of the ejaculate by volume.
   b. Seminal fluid is a complex solution of proteins and enzymes that include the following components:
      1. Acid phosphatase (unique to prostatic fluid)
      2. Citric acid
      3. Zinc
      4. Fructose
      5. Fibrinogen-like coagulable proteins

2. Seminal fluid has the following mechanisms in fertilization:
   a. Activation of spermatozoal motility
   b. Provision of nutritive substances
   c. Conveyance of the spermatozoa to the female egg
   d. Provision of enzymes necessary to penetrate the ovum wall and achieve fertilization

3. Spermatozoa synthesis occurs in Sertoli cells and their maturation takes place in the testes.
   a. Spermatozoa have a haploid complement of chromosomes.
   b. FSH (sometimes called “interstitial cell stimulating hormone”) from the pituitary gland is necessary for the initiation of spermatogenesis. Inhibin (from Sertoli cells) inhibits FSH synthesis.
   c. Testosterone (secreted by Leydig cells of the testes) is necessary for the subsequent stages of sperm maturation.
   d. The stages of cellular maturation from the youngest to the most mature stage are as follows:
      1. Spermatogonia
      2. Spermatocytes
      3. Spermatids
      4. Spermatozoa

B. Indications for seminal fluid analysis

1. Seminal fluid examination should be one of the first tests performed in any infertility investigation.
2. Qualification of donors for artificial insemination programs requires a complete seminal analysis.
3. Analysis can provide the necessary information for the documentation of completeness for a vasectomy.
4. Evaluation of semen quality is necessary for semen and sperm banking.
5. Seminal fluid analysis often is necessary for forensic studies in sexually related crimes, such as rape. Forensic studies of seminal fluid are also used in paternity allegations.

C. Routine analysis of seminal fluid involves a physical, chemical, and microscopic examination of the specimen. Analysis of the specimen should not begin until after liquefaction has occurred.

1. Physical examination includes an assessment of the appearance, volume, and viscosity of the ejaculate.
   a. The normal appearance of semen is gray-white and opalescent, while a brown or red color may indicate blood and a yellowish color can signify lack of ejaculation (abstinence).
   b. Normal volume for an ejaculate is 2 to 5 mL. Volumes outside this range can be associated with infertility.
c. Viscosity can be estimated by observing the formation of droplets that form when the fluid is expelled with a Pasteur pipette.
   (1) A normal semen specimen should liquefy approximately 30 to 60 minutes after ejaculation.
   (2) Increased viscosity is observed when the fluid forms a string as the drop is expelled from the pipette.

2. A microscopic examination is critical for fertility studies and includes a determination of motility, sperm count, sperm morphology, viability, and an examination of cells other than sperm.
   a. Motility is evaluated as an important indicator of fertility. Sperm motility should be assessed on a liquefied sample within 1 hour of collection.
      (1) Motility is directly proportional to the anatomic integrity of spermatozoa as well as to the ability of sperm to fertilize an ovum.
      (2) Sperm are evaluated microscopically in a counting chamber (e.g., Makler chamber, Neubauer chamber). The slide is prewarmed to 37°C, and sperm are examined with a high-dry objective.
         At least 100 spermatozoa in at least 10 different fields are evaluated on a 0–4+ scale. Motility rankings are based using the following criteria:
         (a) 0: Immobile
         (b) 1.0: Mobile; no forward progression
         (c) 2.0: Mobile; slow nondirectional motility
         (d) 3.0: Mobile; moderate linear progression
         (e) 4.0: Mobile; strong linear progression
      (3) Normal motility is defined as 50% or more of the sperm having a ranking of 3.0 or 4.0.
   b. Sperm counts can be obtained from a Neubauer counting chamber using the same method that is used to obtain a manual WBC or RBC count. A 1:20 dilution of the semen sample is made with sodium bicarbonate and formalin; tap water has also been suggested as a diluent. This fixes the sperm (stops motility).
      (1) A normal sperm count is between 20 million and 200 million sperm per milliliter.
      (2) Infertility is highly associated with counts <20 million with 10 to 20 million considered a borderline result.
      (3) To calculate total sperm count, multiply the number of sperm per milliliter by the specimen volume. Use the formulae that are used in the calculation of RBCs or WBCs for calculating numbers of sperm/mL, depending on the number of hemacytometer squares counted (Chapter 3).
   c. Spermatozoa morphology is also routinely assessed (Figure 9–6) (see Web Color Image 9–42).
      (1) Normally, at least 50% of the sperm should have normal morphology.
      (2) More than 50% morphologically abnormal sperm could be related to infertility.
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d. Viability is observed using supravital eosin staining of a sample of fresh semen. A smear is then made of the stained solution.

(1) Viable sperm do not take up the stain, whereas dead sperm have damaged membranes and absorb the stain. One hundred sperm are counted and evaluated.

(2) The percentage of dead sperm should not exceed the percentage of immobile sperm. Normally, 50% or more of the sperm should be viable.

e. The stained seminal fluid smear should also be examined for the presence of cells other than spermatozoa.

(1) An ejaculate may normally contain leukocytes and urethral epithelial cells.

(2) An increase of leukocytes >1 million/mL one million per milliliter of ejaculate is suggestive of an inflammation.

(3) The presence of RBCs or bacteria indicates a pathologic process.

3. A chemical analysis of seminal fluid is of limited usefulness. However, measurement of the pH, fructose level, and acid phosphatase activity can provide diagnostic information.

a. The pH should be obtained from a fresh seminal specimen.

(1) A pH >7.8 could indicate an infectious process.

(2) A pH <7.0 indicates either successful vasectomy or that the seminal fluid is entirely prostatic fluid, which indicates tubule blockage or inflammation.

b. Fructose is produced and secreted by the seminal vesicles.

(1) A fructose level of >13 μmol per ejaculate reflects normal secretory function.

(2) A low fructose value can indicate ejaculatory duct obstruction or pathology of the vas deferens.

c. Acid phosphatase activity can determine secretory function of the prostate gland, because this gland uniquely secretes high levels of this enzyme. Determination of this enzyme in forensic studies can be used to determine the presence of seminal fluid.

d. Citric acid and zinc levels can be determined to evaluate prostate gland function. Low levels of both substances are associated with prostatitis.

XIV. CEREBROSPINAL FLUID (CSF) ANALYSIS

A. CSF location, formation and functions

1. The central nervous system (CNS) is bathed by a network of CSF-filled reservoirs internally and externally. CSF is synthesized by the choroid plexus in the ventricles and circulates out to the subarachnoid spaces surrounding the CNS (brain and spinal cord).

2. The CSF functions to protect and cushion the CNS, provide nutrients to neural tissue, and remove metabolic waste. Approximately 500 mL/day of CSF is produced, with a total volume of 140 to 170 mL in adults.

B. Specimen collection is only for diagnosis or for the treatment of disease.

1. Ventricular puncture to obtain CSF can be performed by a physician in special circumstances. Typically, sterile lumbar puncture between L2 and L3 vertebrae or between the L3 and L4 vertebrae is the most common collection area. This collection taps CSF in the lumbar cistern of the subarachnoid space around the base of the spinal cord where there is no chance of piercing the cord itself.

2. Three specimen tubes are aseptically collected and labeled as 1, 2, and 3. Each tube is uniquely used for specific testing.

a. Tube 1 is used for CSF chemistry analysis. This tube may contain tissue juice and some cells from the spinal tap.

b. Tube 2 is used for microbiology studies.

c. Tube 3 is used for a hemocytometer cell count and cytospin differential. This tube should contain no contaminating cells from surrounding tissue; therefore, any cells found in this tube are intrinsic to the CSF. Physical examination of the CSF sample may be done using this tube.

3. All testing on CSF specimens should be performed immediately upon receipt of specimen because of rapid cellular degeneration.
C. Pathologic diseases detected by and involving the CSF include the following conditions:

1. Subarachnoid or intracerebral hemorrhages (i.e., strokes or trauma)
2. Infections such as meningitis (e.g., bacterial, fungal, parasitic, or viral); abscesses and encephalitis
3. Malignant processes such as primary brain tumors, metastatic tumors with a primary site elsewhere, or leukemias and lymphomas.
4. Multiple sclerosis.

D. Routine CSF analysis typically includes a physical, chemical, and microscopic analysis.

1. Gross examination includes a report of color and clarity of the specimen.
   a. Normal CSF is clear and colorless.
   b. Turbidity is most often produced by the increased presence of WBCs (>200 cells per milliliter), by increased RBCs (>400 RBCs per milliliter), or by microorganisms. Cellular turbidity is known as pleocytosis.
   c. Abnormal specimen color is most commonly caused by a disease process.
      (1) Pink or red CSF is the result of RBC lysis and can be seen 4–10 hours after a subarachnoid hemorrhage. This can also be caused by a traumatic tap.
      (2) Xanthochromic (i.e., yellow) specimens result from the following conditions:
         (a) After pathologic bleeding caused by breakdown of hemoglobin and bilirubin formation in CSF or due to traumatic tap
         (b) When CSF protein is increased >250 mg/dL
         (c) Liver disease caused by increased total bilirubin levels
      (3) Brown CSF specimens are the result of the following conditions:
         (a) Presence of methemoglobin
         (b) Subdural or intracerebral hematoma
         (c) Presence of melanin caused by a melanoma
   d. Distinguishing between pathologic bleeding and traumatic tap is often necessary. The following criteria are used to distinguish between the two:
      (1) A serial decrease in RBCs in tubes 1 to 3 is seen with a traumatic tap.
      (2) A clotted specimen or clumped RBCs on microscopic examination indicates traumatic tap.
      (3) The color of the supernatant of a bloody specimen after centrifugation can be suggestive. A clear supernatant indicates a traumatic tap, whereas a pink, yellow, or brown supernatant indicates pathologic bleeding. However, if a sample contains bilirubin from lysed RBCs that have come from a traumatic tap, it may have a yellowish color.
      (4) A ratio of >500 RBCs for every WBC indicates traumatic tap.

2. Chemical examination can include many analytes, but only a few have any diagnostic value on a routine basis.
   a. Total protein in CSF represents a combination of prealbumin, albumin, transferrin, and trace amounts of immunoglobulin G (IgG).
      (1) Normally, CSF protein ranges between 20 and 50 mg/dL, with albumin representing 50% to 70% of the total.
      (2) A CSF protein level provides information as to the integrity of the blood-brain barrier since large protein molecules are kept out of CSF.
      (3) Increased protein levels in CSF can be the result of the following conditions:
         (a) Contamination with peripheral blood on obtaining the specimen
         (b) Obstruction of CSF circulation (e.g., hydrocephalus or tumor)
         (c) Tissue degeneration
         (d) Increased permeability of the blood-brain membrane caused by drugs, toxins, or infection (meningitis)
         (e) Intrathecal (by the brain) production of protein by tumors
   (4) Recognizing increases in individual protein constituents in the CSF can be important.
      (a) Prealbumin is uniquely found in CSF specimens.
      (b) Any albumin present in a specimen is from the passage of plasma albumin across a damaged blood-brain barrier (formed by tight junctions between
CHAPTER 9 Urinalysis and Body Fluid Analysis

endothelial cells). CSF albumin is increased when the permeability of the blood-brain barrier is compromised (i.e., normal CSF/serum albumin index < 9.0).
(e) IgG is present in trace amounts but can originate from intrathecal produc-
tion in the CNS.
(i) A CSF/serum IgG index < 0.77 is normal.
(ii) An IgG CSF/serum index > 0.77 is highly indicative of multiple sclerosis.
(d) CSF protein electrophoresis can be a useful tool to distinguish protein
content. Abnormal oligoclonal bands in the gamma region of the trac-
ing are comprised of IgG and are highly diagnostic of multiple sclero-
sis.
(e) Myelin basic proteins may be present with multiple sclerosis.

b. CSF glucose is in equilibrium with plasma glucose.
(1) Normal values in the CSF range from 50 to 80 mg/dL or approximately 60%
of plasma glucose levels.
(2) Levels are decreased in bacterial meningitis and fungal infections.
(3) Levels are increased in hyperglycemia and in cases of a traumatic tap.

c. CSF enzyme levels can be detected and are elevated in a variety of pathologic
conditions.
(1) LDH concentrations can be elevated in the following conditions:
(a) Bacterial and viral meningitis
(b) Subarachnoid hemorrhage
(c) Lymphomas
(d) Leukemias
(e) Metastatic tumors
(2) Creatine kinase (CK) levels can be elevated in the following conditions:
(a) Stroke
(b) Multiple sclerosis
(c) Degenerative disorders
(d) Primary brain tumors
(e) Viral and bacterial meningitis
(f) Epileptic seizure
(3) Aspartate aminotransferase (AST) levels can be elevated in the following con-
ditions:
(a) Intracerebral hemorrhage
(b) Subarachnoid hemorrhage
(c) Bacterial meningitis

d. CSF lactic acid concentrations are unrelated to plasma values.
(1) The normal concentration ranges from 10–22 mg/dL.
(2) Lactate is increased with any disorder associated with increased metabolism
or ischemia in the CNS.
e. CSF electrolytes reflect basically the same values as in serum, and their measure-
ment in CSF is of no diagnostic advantage.

3. Cell count in CSF normally reflects a low number of cells (see Web Color Image 9–43).
a. Normally, mononuclear cells (i.e., lymphocytes, monocytes) predominate in a low
concentration of 0 to 10 cells per microliter.
(1) Neutrophils comprise 0% to 6%.
(2) Monocytes comprise 15% to 45%.
(3) Lymphocytes compose the majority of cell types: 40% to 80%.
b. RBCs should be absent. The presence of RBCs indicates either cerebral hemorrhage
or traumatic tap.
c. Cell counts must be performed within 1 hour of collection.
d. If increased WBCs are found, a cytopsin smear is prepared, and the smear undergoes
Wright’s stain to determine a WBC differential count.
e. Any other cell type or a change in differential percent could indicate a serious
disorder.
(1) An increase in neutrophils is indicative of bacterial meningitis.
An increase in lymphocytes can be found in cases of viral, tubercular, or fungal meningitis.

Plasma cells may be found in individuals who have multiple sclerosis or chronic inflammatory conditions.

An eosinophilia may be associated with parasitic or fungal disorders (see Web Color Image 9–44).

Macrophages can be found in CSF in association with hemorrhage. The presence of ferritin granules in the cytoplasm of CSF macrophages may indicate an older or chronic hemorrhagic condition.

Malignant cells can be present as a result of a CNS tumor, metastatic tumor or a leukemic process.

4. Microscopic examination consists of a Gram’s stain or acid-fast stain, as well as a microbiologic culture and sensitivity determination. Viral studies or fungal cultures can also be performed (see Chapter 7).

5. Meningitis-causing organisms include the following:
   (1) Cryptococcus spp.
   (2) Coccidioides immitis
   (3) Mycobacterium tuberculosis
   (4) Haemophilus influenzae
   (5) Neisseria meningitidis
   (6) Streptococcus pneumoniae
   (7) Staphylococcus aureus

**XV. GASTRIC FLUID ANALYSIS**

A. Physiology of gastric secretion and digestion
   1. Three physiologic functions of the stomach include:
      a. Serves as an expandable reservoir for ingested food;
      b. Initiates protein digestion by pepsin;
      c. Secretion of intrinsic factor for binding and absorption of vitamin B12 in the ileum.
   2. Three general phases of gastric secretion include
      a. The cephalic (neurogenic) phase involves vagus nerve stimulation caused by stimuli such as taste, smell, or sight. Anticipation of food (i.e., hunger response) has four effects on the stomach.
         (1) Gastrin is secreted into the blood by specialized G cells in the pyloric gland and stomach and by the delta cells of the pancreas.
         (2) Gastrin induces secretion of hydrochloric acid by parietal cells of the gastric glands in the proximal body and fundus and secretion of pepsin by the chief cells.
         (3) Vagal excitation lowers the threshold of parietal cells to gastrin stimulation.
         (4) Gastric peristalsis and emptying is promoted.
      b. The gastric phase involves stomach distention and continued gastric secretion of digestive juices.
      c. The intestinal phase involves digestion in the small intestines.
         (1) Secretion of gastrin by G cells is inhibited by gastric inhibitory polypeptide (GIP, a member of the secretin family), which is secreted by the duodenal glands in the small intestine. GIP also causes insulin to be secreted and stimulates lipoprotein lipase.
         (2) Gastrin secretion is reduced at a pH ≤ 3.0 and stops completely at a pH ≤ 1.5. When K cells in the distal duodenum and proximal jejunum come in contact with fats, glucose, or amino acids, gastric GIP is produced.

B. Chemical composition of gastric fluid
   1. Hydrochloric acid (HCl) is present in varying amounts.
      a. HCl is secreted by parietal cells in the fundus of the stomach.
      b. HCl converts pepsinogen to pepsin and hydrolyzes polypeptides and disaccharides.
   2. Pepsin, which catalyzes protein breakdown at a pH of 1.6–3.6, is also present.
CHAPTER 9 Urinalysis and Body Fluid Analysis

3. Mucus is present to protect the mucosa of the stomach from autodigestion.
4. Miscellaneous substances are present such as water, various enzymes, proteins, and intrinsic factor.

C. Gastric fluid analysis may be necessary for the diagnosis of digestive disorders or ulcers.
1. Assessment of gastric acidity. Gastric acidity may fall into one of several categories.
   a. Anacidity refers to the failure of the stomach acidity to fall lower than 6.0 in a stimulation test.
   b. Hypochlorhydria refers to the physiologic failure of pH to fall below 3.5, although it decreases 1.0 pH unit or more upon gastric stimulation.
   c. Achromolydria is the physiologic failure of pH to fall below 3.5 or 1.0 pH unit with gastric stimulation.
2. Evaluation of gastric fluid acidity may be performed to determine the proper surgical procedure to be used for peptic ulcer treatment.
3. Gastric analysis may aid in the diagnosis of Zollinger-Ellison syndrome, which usually results from a neoplasm of the pancreas and causes increased gastric secretion of acid, secretory volume, and very high blood gastrin levels.
4. Determination of gastric acid secretion in response to insulin-induced hypoglycemia can be beneficial to assess the completeness of surgical vagotomy.
5. A routine physical evaluation of gastric fluid includes the following measurements:
   a. Appearance (i.e., normal is translucent, pale gray, and slightly viscous)
   b. Volume (i.e., normal is 50 to 75 mL)
   c. Odor (i.e., normal is faintly pungent)
   d. Mucus (i.e., normal quantity varies)
   e. Microscopic examination
   f. Measurement of gastric acid and pH

D. Gastric fluid collection is performed using several methods.
1. The basal gastric secretion test measures fasting levels of gastric production. A Levin tube is placed in the stomach for fluid collection.
   a. The gastric secretion collected represents the amount of secretion during a 15-hour fast.
   b. Four 15- or 30-minute specimens are collected. On each gastric specimen, the volume, pH, titratable acidity, and calculated acid output is determined.
2. Stimulation of fluid formation involves the use of pentagastrin to stimulate maximal secretion.
3. Insulin-induced hypoglycemia test is often used to test for the completeness of vagotomy.
   a. Hypoglycemia stimulates the vagus to stimulate gastric acid secretion within 2 hours after insulin injection.
   b. Blood glucose measurements are also taken every 30 minutes (i.e., preinsulin and postinsulin injection) to make sure that glucose falls to 50 mg/dL or lower.
4. Tubeless gastric analysis is a noninvasive method to determine gastric acidity.
   a. Dianex blue, which is a carboxylic acid cationic resin with an indicator dye (i.e., azure A) coupled to it, is given orally to the individual.
   b. Hydrogen ions in gastric secretion combine with the resin and release the azure A ions, which are absorbed into the bloodstream in the small intestine and excreted in the urine.

XVI. FECAL ANALYSIS

A. Abnormal fecal formation is often secondary to gastrointestinal disease.
1. Diarrhea is defined as an increase in volume, liquidity, and frequency of bowel movements as compared with normal.
   a. Secretory diarrhea is caused by increased intestinal secretion of a solute. This condition can be caused by the following circumstances:
      (1) Endotoxin-producing bacteria
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2. Mucosal damage
3. Drugs (e.g., caffeine, prostaglandins) b. Osmotic diarrhea results from the increased ingestion of osmotically active solutes that draw fluid into the gastrointestinal tract. This is seen in the following conditions or treatments:
   (1) Maldigestion caused by a lactase deficiency or a lipase deficiency
   (2) Malabsorption (i.e., celiac disease, tropical sprue)
   (3) Laxative use
   (4) Parasitic infections
   (5) Small bowel resection
c. Increased intestinal motility is caused by the following conditions:
   (1) Parasympathetic nervous system activity (e.g., stress or stimulation with caffeine)
   (2) Laxatives (e.g., castor oil)
   (3) Cardiovascular drugs (e.g., digitalis)

2. Steatorrhea is defined as fecal fat excretion exceeding 3 g/day.
a. Dietary fat has only a small effect on the total amount of fecal fat.
b. With steatorrhea, feces appear pale, greasy, bulky, spongy, or pasty in consistency and have a very strong odor.
c. Steatorrhea can occur in combination with diarrhea and can result from either maldigestion or malabsorption.
   (1) Maldigestion results from decreased levels of pancreatic enzymes (e.g., pancreatitis, pancreatic cancer) or decreased bile-acid formation (e.g., obstructive jaundice).
   (2) Malabsorption results from diseases that damage intestinal mucosa (e.g., tropical sprue, celiac disease)

B. Specimen collection generally follows rules of common sense.
1. Individuals must be instructed how to obtain a proper specimen.
2. A specimen container can be any clean, nonbreakable, leakproof container that is large enough to contain the specimen.
3. The type and amount of fecal specimen depends on the type of test ordered.
a. For fecal occult blood, WBCs, or qualitative fat, only a small specimen is required.
b. Quantitative fecal fat analysis requires a 72-hour specimen.
4. The technologist must be aware of contaminants such as urine, water, or paper.

C. Macroscopic examination includes observation of specimen color, consistency, and form.
1. Color changes of the stool specimen can result from gastrointestinal irregularities. Color changes of stool and their indications include the following:
a. Brown—normal
b. Gray—intestinal obstruction or barium
c. Red—blood or food dyes
d. Black—blood from upper gastrointestinal tract, iron therapy, antacids, or charcoal treatment
e. Green—vegetables, biliverdin
2. Consistency can demonstrate the following variety:
a. Formed—normal
b. Hard—constipated
c. Watery—diarrhea or steatorrhea
3. Form of the stool specimen and changes in form can also be caused by gastrointestinal irregularities. The types of fecal form and their indications include the following:
a. Cylindrical—normal
b. Ribbon-like—intestinal strictures, such as tumor blockage
c. Small, round—constipation
d. Bulky and frothy—steatorrhea
e. Mucoid—colitis, constipation
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D. Chemical examination typically includes an examination for only blood, fat, or carbohydrates.

1. Fecal blood determination is important as an early detector of colorectal cancer. Other causes of blood in feces include hemorrhoids, anal fissures, colon polyps, ulcerative colitis, and Crohn’s disease.
   a. The physician must be aware that any bleeding from the gums to the anus can result in positive fecal blood.
      (1) Melena is defined as large amounts of fecal blood (i.e., 50–100 mL/d) that turn the stool black.
      (2) Occult (hidden) blood refers to small amounts of fecal blood (i.e., 30–50 mL/d).
   b. Testing principles are designed for sensitivity and turnaround times.
      (1) Hemoglobin-reduction methods are based on the reaction of hemoglobin (Hb) with hydrogen peroxide (H$_2$O$_2$) and an indicator. These methods are popular and very common because of their ease of operation and short testing time. The chemical reaction formula is demonstrated as follows:
         \[ \text{H}_2\text{O}_2 + \text{Indicator} \rightarrow \text{Oxidized indicator} + \text{H}_2\text{O} + (\text{Hb}) \text{ (blue-green color)} \]
      (a) The following are common indicators that are used (most sensitive to least sensitive):
         (i) Benzidine (carcinogenic)
         (ii) Orthotoluidine
         (iii) Guaiac (most common)
      (b) There are a variety of interfering factors that can cause false-positive or false-negative test results.
         (i) False-positive results are seen with a diet of rare-cooked meats; some vegetables (e.g., turnips, broccoli); some fruits (e.g., cantaloupes, bananas); and drugs (e.g., aspirin).
         (ii) False-negative results can be caused by vitamin C (ascorbic acid).
      (c) There are limitations to the sensitivity and specificity of this method.
         (i) More than one fecal site needs to be tested for accuracy.
         (ii) If Hb has been degraded on standing or in the gastrointestinal tract, its pseudoperoxidase activity has been lost, and it will not react with the indicator.

2. Fetal hemoglobin (Hb F) in feces must sometimes be determined, because differentiation between fetal and maternal blood is critical.
   a. Newborns may excrete stools (meconium) containing blood and other substances originating from maternal blood that was ingested during delivery.
   b. Maternal RBCs can be distinguished from fetal RBCs in a stool sample with the Apt test.
      (1) The test principle is based on the alkaline resistance of fetal Hb.
      (2) A fecal suspension is made, and 5 mL of the supernatant is mixed with 1 mL of 0.25 mol/L sodium hydroxide.
   c. If the pink color of the blood changes to yellow or brown in 2 minutes, the Hb present is Hb A (i.e., adult, maternal).
   d. If the pink color remains, the Hb present is Hb F.

3. Quantitative fecal fat is a definitive test for steatorrhea.
   a. Two days before collection, the individual is put on a normal diet to include adequate fat and caloric intake.
   b. Typically, a 72-hour sample is needed.
   c. The test principle is based on the determination of fat content by either titrimetric or gravimetric methods.
   d. The procedure involves first weighing the sample and then homogenizing it.
      (1) Analysis by the titrimetric method involves the following basic steps:
         (a) Lipids are converted to free fatty acids before extraction with a solvent.
         (b) The extracted free fatty acids are titrated against sodium hydroxide.
         (c) This method measures up to 80% of the total fat content.
The gravimetric method of analysis quantitates up to 100% of fats.

Percent fat retention can be calculated using the following formula:

\[ \text{% Fat retention} = \frac{\text{Dietary gram fat} - \text{fecal fat}}{\text{Dietary gram fat}} \times 100 \]

4. Fecal carbohydrate analysis may be necessary to differentiate disaccharidase enzyme deficiencies.

a. If disaccharides from the diet are not enzymatically reduced to monosaccharides, they will remain in the intestine and be osmotically active, producing an osmotic diarrhea.

b. Causes of osmotic diarrhea due to an increase of disaccharides in the gastrointestinal tract include the following disorders:
   (1) Hereditary disaccharidase deficiency is rare.
   (2) Acquired disaccharidase deficiency occurs from malabsorption diseases (e.g., tropical sprue) or from drug effects (i.e., neomycin).
   (3) Lactose intolerance is the most common cause, and it is seen most often in African or Asian populations.

c. Osmotic diarrheal stools characteristically have an acid pH between 5.0 and 6.0. Normal feces are alkaline (i.e., >7.0).

d. Analysis for disaccharidase deficiency includes the following testing methods:
   (1) Clinitest can be used to test for reducing sugars, but the specific disaccharide is not identified, and sucrose cannot be detected.
   (2) Determination of specific enzyme deficiencies can be made by an oral tolerance test using specific sugars (e.g., lactose, sucrose).
      (a) Normally, the disaccharide is converted to a monosaccharide (e.g., glucose, galactose) in the small intestine.
      (b) An increase in the individual's blood glucose or galactose 30 mg/dL greater than the fasting level indicates adequate enzyme activity.

E. Microscopic examination of feces is necessary to identify stool WBCs, the qualitative presence of increased fecal fat, and meat or muscle fibers.

1. A determination of fecal WBCs aids in the differential diagnosis of diarrhea. A smear is made on a glass slide and stained with Wright's stain.

a. The presence of stool WBCs, especially neutrophils, indicates an infectious or inflammatory intestinal mucosal wall. Bacteria that cause increased fecal WBCs include Salmonella, Shigella, Campylobacter, Yersinia, and E. coli.

b. The finding of stool WBCs can indicate the following disorders:
   (1) Ulcerative colitis
   (2) Dysentery (bacterial)
   (3) Ulcerative diverticulitis
   (4) Intestinal tuberculosis
   (5) Abscesses

2. The presence of fecal fat can also be qualitatively determined microscopically.

a. Sudan III, Sudan IV, or oil red O stains all stain fecal triglycerides orange to red in suspension.
   (1) A suspension of the fecal preparation can also be placed on a slide with several drops of ethanol.
   (2) Stain is added to the slide, and the wet preparation is shielded with a cover slip and observed for stained fat globules.

b. Differentiation. Cases of maldigestion can be differentiated from malabsorption by comparing results from both slide preparations (Table 9–9).
Table 9–9 Maldigestion and Malabsorption Differentiation

<table>
<thead>
<tr>
<th>Cause</th>
<th>First Slide (Neutral Fat)</th>
<th>Second Slide (Free Fatty Acids)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malabsorption</td>
<td>Normal</td>
<td>Increased</td>
</tr>
<tr>
<td>Maldigestion</td>
<td>Increased</td>
<td>Normal or increased</td>
</tr>
</tbody>
</table>

3. The increased presence of meat fibers can be diagnostically significant.
   a. An increase in fecal meat fibers (cretorhea) indicates impaired digestion and/or rapid intestinal transit. This indicates pancreatic insufficiency, gastrocolic fistulas, or biliary obstruction.
   b. Meat fibers are identified microscopically as rectangular or cylindrical fibers with cross striations.

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- Web Color Images 9–1 through 9–44
- Study Questions and Answers
CHAPTER 10

Molecular Diagnostics

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I. BIOLOGY OF THE CELL

A. Organelles. In contrast to bacterial cells, all eukaryotic cells contain membrane-bound organelles.

1. Lipid bilayer
   a. Cell membrane functions as the “shell” of each cell in the body; contains a hydrophilic and a hydrophobic surface; and encloses the contents of the cell.
   b. Membrane bound organelles within the cell are encased in a lipid bilayer.

2. Nucleus is the structure containing the majority of genetic material within the cell.
   a. DNA is the genetic material of the cell. It is organized into chromosomes.

3. Mitochondria are numerous within each cell. The number varies by cell type.
   a. Energy generation occurs within mitochondria.
   b. Oxidative phosphorylation

4. Endoplasmic reticulum is a structure that is continuous with the nucleus, but contains no DNA.
   a. It is the site of protein synthesis.

5. Golgi apparatus
   a. Protein modification
   b. Protein transport to the cytosol

6. Lysosomes and peroxisomes
   a. Function in the removal of waste products within the cell

B. DNA is the genetic material of all cells and 99% is contained in the nucleus.

1. Structure
   b. A phosphodiester backbone links the bases together to form a chain.
   c. A deoxyribose sugar is also contained at each base position.
   d. Double helix. Two DNA chains are hydrogen bonded to one another between adenine and thymine or cytosine and guanine. The two joined, antiparallel strands then twist into a double-helix (Figure 10–1).
   e. Genes. Along the DNA strands are specific, short regions that encode for genes.

2. Chromosomes. Human DNA is further arranged into 23 pairs of matched chromosomes.
   a. Chromatids. The DNA strands are wound around histone proteins and these coil together to form a tightly packed chromatid.
   b. Centromeres. The two sister chromatids are joined at the centromere. These two joined chromatids form a chromosome.
c. Telomeres. The ends of chromosomes contain repetitive sequences of a specific 6-base sequence: TTAGGG. This repeated sequence can be many hundreds of bases long. Telomeres protect genes at the ends of chromosomes from normal damage and shortening during DNA replication. They are also thought to be involved in the aging process.

3. Plasmids
a. Extrachromosomal, circular pieces of DNA first identified in bacterial cells
b. confer antibiotic resistance to bacteria
c. Engineered in the laboratory to clone specific pieces of human DNA

4. DNA Replication
a. Semiconservative process
b. Strands are separated by enzymes and new nucleotides added to form a new strand using the original strand as a template. Replication is carried out by DNA polymerases.
c. The result is the generation of two identical strands of DNA, each of which contain one strand from the original duplex.
d. Carried out in the 3-prime to 5-prime direction. One strand is directly copied from its template. The other strand is replicated in small pieces known as Okazaki fragments. These fragments are later joined by DNA ligase to form a contiguous strand of DNA.

C. RNA
1. Classes
a. tRNA. Defines amino acid to be incorporated into a polypeptide
b. rRNA. Forms a portion of the ribosome and participates in protein synthesis
c. mRNA. Template from which a polypeptide or protein is made

2. Transcription is the process of converting the genetic code contained within the DNA into an RNA copy. After this process, the mRNA copy undergoes specific post-translational modifications to prevent degradation, function in controlling the rate of protein synthesis, repair misincorporated nucleotides, or increase genetic diversity.

a. Polyadenylation. Addition of long sequence of A nucleotides to the 3’ end of mRNA
b. Capping. Addition of a methyl group to the 5′ end of mRNA
c. Splicing. Inserting, deleting, or editing nucleotides after synthesis

D. Proteins
1. Translation is the process of utilizing mRNA transcripts to produce a polypeptide (or protein) represented by the original genetic code.
   a. The sequence of an mRNA is converted to a polypeptide.
   b. Occurs in the ribosome
2. Amino acids
   a. Represented by a triplet codon sequence
   b. Basic components of proteins
   c. Contain an amino and a carboxyl group
   d. Twenty amino acids found in proteins
3. Polypeptides
   a. Chain of amino acids
   b. α-helix or β-sheet
   c. Component or subunit of a mature protein
4. Enzymes
   a. All are proteins
   b. Catalyze specific chemical reactions within or outside of the cell
   c. DNA synthesis proteins are common examples of enzymes
   d. Degradation of cellular components
   e. Cell division
   f. Enzymes are also produced commercially for use in the laboratory.

II. MOLECULAR DIAGNOSTIC METHODS

A. Nucleic acid isolation
1. Specimen collection
   a. Whole blood. This is the most common type of molecular diagnostic specimen. Blood is most commonly collected in purple-top tubes. Heparin is a potent inhibitor of PCR and is, therefore, not used in molecular diagnostics. Blood should be stored at 4°C until processing.
   b. Tissue. Tissue specimens should be frozen immediately upon receipt or held in cell culture medium at 37°C until processing.
   c. Buccal swabs. Often used for remote site testing; collection of DNA specimens for parentage testing. Swabs should be air-dried before shipment to the laboratory.
   d. Other specimens include those also commonly encountered in the clinical lab including urine, feces, sputum, cerebrospinal fluid, genital swabs, semen, etc. Other molecular diagnostic specimens include sections of formalin-fixed paraffin-embedded tissues, bone, hair, and fingernails (usually confined to forensic specimens).
2. DNA isolation
   a. Phenol:Chloroform:isoamyl alcohol method is the “gold standard.” Yields a large mass of high quality DNA. Can be used to isolate DNA from any specimen type. This method normally requires a large mass of starting material.
   b. Manufacturer (Qiagen) kits. Provide ease of use and high degree of reproducibility. Yields smaller amounts of DNA. DNA is usually very pure. Most often used to isolate DNA from blood. This is the most commonly encountered clinical DNA isolation method.
   c. Quick-extract solutions. Fastest methods. Low quality, low mass DNA recovery. Usually used to isolate DNA from buccal swabs/FTA cards.
3. RNA isolation
   a. Acid phenol/LiCl salt isolations. Gold standard. Yields a large mass of high quality RNA. Can be used on any specimen type.
b. Manufacturer kits. Provide ease of use and high degree of reproducibility. Preferred method for clinical RNA isolation.

c. Poly-A enrichment kits. Increase recovery of mRNA in specimens.

4. Spectrophotometry

a. A260 value is used to calculate nucleic acid mass.

b. DNA A260/280 ratio between 1.8 and 2.0 is indicative of DNA quality sufficient for clinical manipulation. Lower ratios indicate impure specimens.

c. RNA A260/280 ratio between 1.9 and 2.1 is indicative of RNA quality sufficient for clinical manipulation. Lower ratios indicate impure specimens.

B. Nucleic acid amplification

1. Target amplification

   a. Polymerase chain reaction (PCR) is the method by which almost all molecular diagnostic assays are performed. It is a three-step process consisting of melting the duplex DNA strands, annealing the primers, and extending the new fragment to yield a specific, short DNA product. This process is repeated 25 to 40 times to theoretically yield trillions of copies from one starting copy of DNA (Figure 10–2).

   (1) Primers are short segments of synthetically manufactured DNA used to define a specific position to be copied in the PCR process. Each PCR reaction requires two primers—one on either side of the fragment to be amplified.

   (2) Magnesium chloride is a salt required as a co-factor for the polymerase (enzyme) used to carry out PCR.

   (3) Taq polymerase is the enzyme used to generate new DNA copies in the PCR process. It is a thermostable enzyme, meaning that it will not degrade at very high temperatures used in the PCR process.

   ! Figure 10–2 Polymerase chain reaction. A: The target DNA sequence is indicated by the bold line. B: The double stranded DNA is denatured (separated) by heating. C: Reagents are added, and the primer binds to the target DNA sequence. D: polymerase extends the primers. E-G: Heating, annealing of the primer, and extension are repeated. (Reprinted from Bishop ML. Clinical Chemistry. 5th ed. Baltimore: Lippincott Williams & Wilkins; 2005 with permission.)
Buffers containing potassium chloride, sodium chloride, and other additives are also included in the reaction to stabilize the enzymes and DNA during the PCR process.

dNTPs (A, T, G, and C) are the individual building blocks of the DNA strand.

A very small mass of the genomic DNA specimen of the patient to be tested is also added to the reaction. The PCR process only requires femtomolar quantities of input DNA.

Transcription-based amplification (TMA) is an alternative method to PCR that is not based upon direct amplification of the genomic DNA template.

Probe amplification is another method that does not rely on direct amplification of the template DNA strand. Two commonly encountered clinical examples of this strategy include:

Ligase chain reaction

Multiplex ligation-dependent probe amplification (MLPA). This method is used clinically to detect deletions of multiple entire exons in very long stretches of DNA representing large genes.

Strand displacement amplification

Signal amplification is yet another method of detecting a DNA target without direct amplification of the template DNA. It relies on detection of substrates bound to the target nucleic acid.

Branched DNA amplification

Hybrid capture

Restriction digestion is a method used in many applications within the molecular diagnostics laboratory. It is a method used to fractionate DNA at sequence-specific locations using restriction enzymes. Restriction digestion is used to generate size standards and to prepare genomic DNA for analysis by Southern blotting and hybridization.

Southern blot is a method to analyze the DNA contained in an entire genome through a process of restriction digestion, electrophoresis, transfer to a membrane, and hybridization to a sequence-specific probe.

Northern blot is analogous to the process of Southern blotting, but is performed on RNA specimens.

Probes are small fragments of DNA used to detect a specific gene or mutation in the process of Southern or Northern blotting.

Hybridization dynamics are conditions, such as salt concentration, temperature, time and buffer composition, that control the stringency of the hybridization procedure.

Gel electrophoresis is a method used to separate DNA fragments onto a solid matrix based upon size.

Agarose gel electrophoresis is most often performed to resolve DNA fragments larger than 100 bp in size.

Polyacrylamide gel electrophoresis is the method of choice to resolve small DNA fragments. Very high concentrations of polyacrylamide matrix can resolve down to 1 bp differences in size.

Capillary gel electrophoresis is a modification of polyacrylamide gels used in automated DNA sequencing and genotyping platforms.

Quantitative real-time PCR is a PCR method starting the mRNA as a template. This method is capable of quantifying the copy number of mRNA transcripts present in a specimen.

Reverse transcriptase is the enzyme used to make a copy DNA, or cDNA, from the mRNA transcript contained in a specimen. This process is referred to as cDNA synthesis.

Real-time PCR is the general term for the PCR method used to observe the production of new DNA fragments in real time on an automated platform.

SYBR green is a dye often used in quantitative real-time PCR assays to detect newly generated DNA fragments.
b. **TaqMan** chemistry is an alternate system of amplification and detection of fragments in quantitative real-time PCR using dye-labeled probes.

G. **DNA sequencing** allows determination of each base pair in a single strand of DNA.
   1. **Sanger dideoxy sequencing** is the original method of DNA sequencing and the basis of all DNA sequencing used in the clinical environment today. It is based upon the incorporation of dideoxy nucleotides in random positions in the newly synthesized DNA strand.
   2. **Fluorescent DNA sequencing** is a method by which DNA sequence is determined based upon incorporation of a dye-labeled dideoxy nucleotide and resolution on a capillary electrophoresis instrument. Each dideoxy nucleotide is labeled with a specific dye and when incorporated, is excited by a laser and detected by a CCD camera on an automated platform.

H. **Microarray** technologies are the newest area of use in molecular diagnostics. They fall into two general categories:
   1. **Single nucleotide polymorphism** arrays are those designed to detect single base or other small changes in DNA sequence across the entire genome simultaneously. Many thousands of positions can be interrogated in one experiment.
   2. **Gene expression** arrays are those designed to assess global changes in expression of specific genes in a given tissue at a given time or under a specific set of treatment parameters.

### III. INHERITED GENETIC DISEASE

A. **Fragile-X** is characterized by the following:
   1. Trinucleotide repeat expansion
   2. X-linked
   3. Mental retardation
   4. Primary affects males

B. **Duchenne muscular dystrophy** is characterized by the following:
   1. Deletion of entire exons
   2. Dystrophin gene
   3. X-linked
   4. Muscle degeneration

C. **Myotonic dystrophy** is characterized by the following:
   1. Trinucleotide repeat expansion with anticipation (the worsening of clinical symptoms with increasing repeat size in successive generations)
   2. Autosomal dominant
   3. Males and females
   4. Multisystem disease

D. **Cystic fibrosis (CF)** is characterized by the following:
   1. Many individual mutations in the CFTR gene may give rise to CF alone or in combination.
   2. Delta F508 mutation accounts for over 70% of CF cases.
   3. Autosomal recessive
   4. Although it primarily affects the lungs, it is a multisystem disease.
   5. Most common genetic disease in Caucasians

### IV. MOLECULAR ONCOLOGY

A. **Minimal residual disease** detection is a commonly performed assay in molecular diagnostics. It is the determination of the effectiveness of a therapy or the tracking of a patient’s
disease status based upon the presence of specific mRNA transcripts known to cause leukemia or lymphoma.

1. **Chronic myeloid leukemia (CML)** is characterized by the presence of the Philadelphiachromosome or 9;22 translocation. The transcript can be detected by quantitative real-time PCR and the size determined by fluorescent capillary electrophoresis.

B. In molecular oncology, **clonality** is the presence of a specific T or B cell clone originating from a lymphoma. The clone is detected using PCR and fluorescent capillary electrophoresis.

C. **Microsatellite instability** is a genetic phenomenon observed in cells undergoing changes in specific cells. It is the expansion of short, repetitive stretches of DNA to much longer stretches that change gene expression within the cell.

1. **HNPCC** is a hereditary form of colon cancer that is marked by microsatellite instability.

D. **Loss of heterozygosity** is the nature of some tumors to exhibit loss of function of one allele in a specific gene in a cell in which the other gene was already inactivated. The most common example of a cancer caused by this process is **retinoblastoma**, in which expression of a tumor suppressor gene is lost.

E. **Drug metabolism and sensitivity** can be determined through genotyping of DNA or gene expression analysis.

1. **Cytochrome P450 (P450)** genotyping is performed to determine drug sensitivity. The test is carried out on a microarray platform.

**V. MOLECULAR INFECTIOUS DISEASE**

A. **Bacterial** identification. The following organisms can be easily detected (qualitative identification) using PCR:

1. *Chlamydia trachomatis*
2. *Neisseria gonorrhoeae*
3. MRSA
4. VRE

B. **Viral** identification may also be performed in a quantitative manner. The following may be quantitatively detected:

1. **Viral load**—Quantitative
   a. HCV
   b. HIV
   c. CV

**VI. DNA-BASED HUMAN IDENTIFICATION**

There are a number of ways by which human identification may be performed. The most widely used system is based upon determination of the length of repetitive DNA in specific regions of chromosomes across the human genome. This is termed **DNA-based human identification**.

A. **HLA polymorphisms** are not as diverse as those observed using other systems of identification. This is an older system of human identification.

B. **RFLP analysis** was the first DNA-based identification system. It is based upon polymorphism in the length of restriction enzyme-cut products across the genome. It utilizes Southern blotting and hybridization as a method of laboratory testing.

C. **STR analysis**

1. Most widely used system for identification of individuals
2. Very high power of discrimination
3. Common alleles are typed and compared to the CODIS database for criminal investigations.
4. Forensic DNA typing of older specimens or “cold cases” utilizes this system when the specimens are in acceptable condition.
5. Parentage testing uses the same STR system as those used in the CODIS allele database.
6. Bone marrow engraftment is a clinical application of this chemistry.
   a. STR analysis may be performed to track donor versus recipient DNA components following transplant.
7. Other uses of DNA-based human identification include QC of histological specimens to ensure that a patient matches a specific specimen.

D. Mitochondrial DNA analysis is identification of individuals based upon their mitochondrial haplotype.
   1. Primarily used on degraded specimens
   2. Only capable of determining maternally inherited alleles with this system, because mitochondria are passed from mothers to all offspring.
   3. Often used in “cold cases,” because there are many hundreds of copies of mitochondrial DNA present for every one copy of nuclear DNA. This allows for a greater possibility of success in analysis.

E. Y-chromosome analysis is a newer system for human identification.
   1. Only capable of determining paternally inherited alleles
   2. System only used to type males
   3. Based upon repeat length polymorphism

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CHAPTER 11
Current Issues in Laboratory Management

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I. MANAGEMENT AND ORGANIZATIONAL THEORY

A. Management (administration) can be defined as: the organizing and controlling of the affairs of a business or a sector of a business or “working with and through people to accomplish a common mission.”

B. Management/leadership theories. One factor that impacts leadership success is management style. There are primarily five leadership theories:

1. Tannenbaum and Schmidt theory. Styles can be plotted on a continuum from authoritative to democratic.
2. McGregor X and Y theory. McGregor X theory relates to authoritative leader, whereas the Y theory relates to the democratic leader.
3. Blake and Mouton theory. Describes five types of management situations:
   a. Impoverished management. Low concern for people and production
   b. Authority-compliance. Low concern for people, but high concern for production
   c. Middle-of-the-road management. Medium concern for people and production
   d. Country club management. High concern for people, but low concern for production
   e. Team management. High concern for people and production
4. Fielder theory. This theory indicates that the style used by the leader may vary according to the situation with a very favorable or very unfavorable situation requiring a task-oriented leader and a moderately favorable or moderately unfavorable situation requiring a relationship-oriented leader.
5. Hersey-Blanchard theory. Four leadership situations:
   a. Employee new to job
   b. Employee has mastered some of the job, but needs supervision.
   c. Employee has mastered the job, but needs verification.
   d. Employee has mastered the job and is confident.

C. Skills of managers

1. Organizational skills. Conceptualize and apply management process, systematize work flow, make decisions, and communicate with coworkers.
2. People skills. Understands basic theories of human needs and work motivation.

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3. Financial skills. Effective use of and accounting for the monetary assets of the company.

4. Technical skills. Involve the synthesis of the first three skills above and the management of physical resources (supplies, equipment, facilities) into the operational parameters (products and services).

D. Management theories

1. Scientific management. Attempts to apply a systematic or scientific approach to the study of organizations developed by Frederick Taylor, the father of scientific theory. Applies cause-and-effect analysis, breaking down each task into segments to improve efficiency.

2. Bureaucracy management. Examines the organizational aspects of the company and its work flow to explain how institutions function and how to improve their structural process. Hierarchical chain of command is an example. Special attention is given to the bureaucratic characteristics as they relate to rules, regulations, impersonality, and division of labor.

3. Behavioral science. Focuses on the performance and interaction of people within the organization by utilizing psychology and sociology, as well as management theories.

4. Systems analysis. An outgrowth of management science, which views the organization as a continuous process interacting within itself and with its environment.
   a. Involves Peter Drucker’s five basic management operations:
      (1) Setting objectives
      (2) Organizing
      (3) Motivating and communicating
      (4) Establishing standards (measurement of performance)
      (5) Developing people, including managers

E. Management process. The concept of management as a continuous process of interacting functions, each dependent on the success of the other.

1. Four main functions:
   a. Planning
   b. Organizing
   c. Directing
   d. Controlling

F. Management by objectives (MBO). A program that embodies all of the concepts in the management process (planning, operating, directing, and controlling). First introduced by Peter Drucker in the 1960s. The core of MBO is communications. Success or failure depends on how well employees understand the company’s mission and objectives. Managers who allow employees to share in the decision-making process will have effective MBO programs. Managers must understand and be skilled at writing and monitoring objectives.

G. Motivation. The driving forces that make people act as they do. This also can be defined as incentives, motives, purpose, drive, desires, and needs. Includes three major theories:

1. Maslow’s hierarchy of needs. Identified physiological and safety needs as basic physical needs that would motivate if not satisfied. Satisfaction of physical needs is required before higher level social need, such as esteem and love, will motivate

2. Herzberg’s two-factor theory. Herzberg (1959) defined two factors:
   a. The basic needs, hygiene factors, which correspond primarily with Maslow’s physical and social needs.
   b. Motivators correspond primarily with Maslow’s growth needs.

3. Vroom’s expectancy theory. Effort will bring reward. The effort expended will be related to the perceived value of the reward. People make a choice between alternative courses of actions according to how they perceive the resulting benefits.
II. PROFESSIONALISM

A. Definition. Professionalism is judged by the professional quality with which one practices the profession and includes areas such as altruism, honor, integrity, caring, compassion, respect, responsibility, accountability, scholarship, and leadership.

B. Definition. Professional ethics is the study of standards of conduct and moral judgments which provide a basis for right and wrong conduct. The American Society for Clinical Laboratory Science (ASCLS) has developed and published the Code of Ethics for Clinical Laboratory Science comprised of duty to the patient, duty to colleagues, and duty to society.

C. Job Description
   1. Purpose. The job description defines the functions and purpose of a specific position for an employee.
   2. The job description should include:
      a. Name of organization
      b. Job title
      c. Summary of duties for the position
      d. Essential functions required for the position
      e. Immediate supervisor (chain of command)
      f. Working conditions/environment
      g. Required qualifications and experience

D. Resume
   1. A resume is a tool for a candidate to demonstrate their strengths, education, and accomplishments when seeking employment. The resume should include the following:
      a. Candidates name (legal)
      b. Contact information (including e-mail)
      c. Career objective
      d. Educational qualifications
      e. Clinical experience
      f. Military experience
      g. Certifications
      h. Professional society memberships
      i. Honors
      j. Publications/research activities/special projects
      k. Volunteer work
      l. Statement regarding references
   2. A cover letter should accompany a resume to focus attention on the applicant. It is best to address the cover letter to a specific individual.

E. Employee Interview and selection
   1. The purpose of the interview process is for the employer to confirm that the provided, written information gathered from the resume and/or job application is valid. Additionally, the interview provides the employer with insight into the candidate’s interpersonal communication skills. The interview also provides the candidate with the opportunity to discuss information in more detail and to ask questions pertinent to the specific position.
   2. The selection process should be conducted after the interview and should include an evaluation of the candidate’s resume/application and information from the interview. The manager is seeking to select the candidate with the appropriate skills, certifications, and personality to fit the institution and the position.

F. Employee evaluation
   1. The employee evaluation process is a method of recognizing job excellence and determining areas for development and training. The job evaluation is directly linked to the
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job description and should be formative and summative in nature and conducted often enough to provide feedback and direction for the employee.

a. Criteria-based evaluations indicate specific performance expectations that are linked to the job description.

b. Competency-based evaluations are used to validate competency of skills.

g. Corrective action

1. The purpose of corrective action is to provide the employee with specific goals to enhance their effectiveness and performance within the institution.

a. The first step is verbal counseling to make the employee aware of the situation and to open the lines of communication.

b. The second step is to issue a verbal warning that specifically involves stating the problem, performance in question, and offering resources and assistance (process is documented).

c. The third step is a formal written warning that states the problem, expected improvement in performance, and a timeline for re-evaluation of performance. This document is signed by the immediate supervisor and employee. Failure to meet the stated requirements typically results in dismissal of the employee.

d. Most institutions have a policy that allows the employee to seek an appeal of the situation to a higher level.

h. Communication and interpersonal relationships

1. Communication is an ongoing process of interpreting symbols, decoding speech and phrases, interpreting ideas and feelings, and encoding that information to formulate an appropriate response. Communication typically serves the following purposes:

a. Exchange of knowledge

b. Expression of feelings

c. Expression of ideas and thoughts

d. To attempt to influence another’s thoughts and actions

e. To meet social expectations

2. Barriers to effective communication are:

a. Nonverbal expressions—body language

b. Cultural differences—unfamiliar phrases

c. Paraverbal issues—tone and pitch of voice

d. Environmental—temperature, neatness of appearance

e. Overcommunication—too much information offered at one time

f. Undercommunication—not enough information provided for understanding the issue

g. time issues

i. Team building is essential for optimal employee performance and effectiveness of the section, department, and institution.

1. Tuckman (1965) suggests the following phases of team development:

a. Identifying the team members

b. Team members selecting their roles

c. Determining standards of behavior and expectations

d. Working together towards a common goal

III. QUALITY ASSURANCE

A. Introduction

1. Quality assurance (QA) is a comprehensive set of policies, procedures, and practices that are followed to ensure that a laboratory’s results are reliable. QA evaluates the quality of the services provided. QA provides a way to prevent problems as well as deal with problems that occur. It differs from quality control (QC) in that QC is
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1. Quality control (QC) includes record keeping, maintenance and calibration of equipment, proficiency testing, quality control, and training of personnel. QA is a commitment to quality. Physician and patient relations are a part of this plan.

B. Quality assurance plan
1. All policies and procedures are in writing and available.
2. Staff performance is routinely evaluated.
3. Continuing education is used and documented.
4. A safety program is in place for both staff and patients.
   a. Occupational Safety and Health Administration (OSHA) standards are met.
   b. Hazardous materials are identified and labeled.
   c. Material Safety Data Sheets (MSDS) are available for each hazardous material.
   d. Employee safety training is documented.
   e. Records of any accidents (e.g., needle stick) are kept and reported to appropriate authorities.
   f. Hazardous waste is properly disposed.
   g. Universal precautions are strictly followed.
   h. Hazardous materials are identified and labeled.
   i. Material Safety Data Sheets (MSDS) are available for each hazardous material.
   j. Employee safety training is documented.
   k. Records of any accidents (e.g., needle stick) are kept and reported to appropriate authorities.
   l. Electrical checks of all equipment are routinely performed.
5. Specimen collection and handling (pre-analytical) as well as correct patient preparation follows written guidelines and is documented.
6. A quality control (QC) program is in place (analytical). This ensures that the instruments, reagents, and personnel are functioning properly. A QC program comprises several areas:
   a. A written QC program is required that specifies:
      (1) Frequency of performing controls
      (2) Number of controls to be used
      (3) Type of controls
      (4) Acceptable limits for control results
      (5) Corrective action required if controls are out of range
   b. QC plots of control results are used to detect shifts (a sudden upward or downward change of four or more consecutive values) and trends (the tendency of results to gradually increase or decrease over a period of time).
   c. Equipment maintenance is performed and documented.
   d. Temperatures of incubators, certain instruments, and the room are noted and documented.
7. Checklists are used to ensure that scheduled activities and duties are performed.
8. The laboratory must participate in and document results of proficiency testing (PT). This allows comparison of results from a number of laboratories. Specimens from PT sponsors are to be treated exactly like patient specimens.
9. The laboratory must be accredited by an appropriate agency. This gives official approval and states that the laboratory follows all of the guidelines set forth by the accrediting agency. A laboratory should have a copy of the Laboratory Accreditation Manual on file in the laboratory.

IV. LABORATORY REGULATIONS

A. Clinical Laboratory Improvement Amendments of 1988 (CLIA 88)
1. Background. CLIA 88 is intended to establish regulations for all laboratories, regardless of size or location, where clinical testing is performed for the purpose of diagnosis, treatment, or monitoring of patients’ health. The law was published in the February 28, 1992 issue of the Federal Register. The law became effective for all laboratories on September 1, 1992.
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1. Modifications to the law list the complexity of tests.
2. Laboratory accreditation
   a. All laboratories must have a CLIA certificate to perform testing and receive federal reimbursement.
   b. Certification is based on the level of test complexity.

   (1) Sites performing only waived tests comprise 58% (105,138) of the approximately 180,000 laboratory testing sites in the United States. Waived testing performed in these sites is often wellness testing, screening tests, or other critical testing that introduces a large population of persons into the health care setting. Although the testing performed in Certificate of Waiver sites accounts for <10% of the total U.S. testing volume, this percentage has been increasing each year since the CLIA program began. Types of direct, unprocessed specimens suitable for waived testing are listed below:
   a. Whole blood (fingerstick or anticoagulated blood collected via venipuncture)
      i. Glucose, cholesterol, prothrombin time, infectious mononucleosis, erythrocyte sedimentation rate, hemoglobin-copper sulfate method, spun hematocrit HIV antibody
   b. Urine
      i. Dipstick urinalysis and pregnancy test (hCG)
   c. Throat swab
      i. Group A streptococcal antigen
   d. Nasopharyngeal swab
      i. Influenza
   e. Stool
      i. Occult blood
   f. Saliva
   g. Alcohol
   h. Oral fluid
      i. HIV antibody
   i. Gastric biopsy
      1. H. pylori

(2) Provider-performed microscopy (PPM) includes:
   a. Wet mount, including preparations of vaginal, cervical or skin specimens
   b. Potassium hydroxide (KOH) preparation
   c. Pinworms slides
   d. Fern test
   e. Urine sediment
   f. Vaginal smears
   g. Nasal smear examinations for granulocytes
   h. Fecal leukocyte examinations
      i. Semen analysis
      ii. Qualitative examination
      iii. Presence or absence
      iv. Motility

(3) Moderately complex testing includes:
   a. Hematology
   b. Chemistry
   c. Serology
   d. Coagulation
   e. Therapeutic drug monitoring
   f. Blood gases
   g. Urine colony counts
   h. Blood type and Rh factor
   i. Limited immunology
   j. Manual differential (limited identification; normal cells only)
   k. Gram’s stain
(i) Urethral for gas chromatography (GC)
(ii) Cervical for GC
(l) Throat culture screen only
(i) Hemolysis
(ii) Bacitracin
(iii) Strept select agar
(m) Microscopic urinalysis (non-PPM)
(n) Rapid kits

[4] Highly complex testing includes:
(a) Microbiology
(b) Immunohematology
(c) Bacteriology
(d) Cytology
(e) Histopathology
(f) Mycology
(g) Manual hematology counts
(h) Manual differential
   (i) Identification of abnormal cells
   (ii) Complete identification
   (iii) Nonautomated chemistry tests

c. The certificate fee and biennial inspection fee, which are based on the size of
   the laboratory and number of tests performed annually, must be paid.
d. Accreditation by a private organization is an alternative option for certifica-
   tion.
   (1) The Health Care Financing Administration (HCFA) may deem a laboratory to
       meet all of the CLIA requirements if the laboratory is accredited by a private,
       nonprofit organization that is certified as having deemed status. This means
       that the certifying agency must:
       (a) Provide reasonable assurance to HCFA that it requires the laboratories it ac-
          credits to meet all of the requirements equivalent to the CLIA requirements
           in the law
       (b) Inspect its laboratories per condition level requirements of the law

   (2) Approved state laboratory programs are responsible for accreditation of lab-
       oratories in their state, if this is a requirement of the department of health of
       that state.

3. Personnel requirements must be met by laboratories performing moderately or highly
   complex testing. These positions can be held by the same person or by different persons
   who meet the qualifications.
   a. Moderately complex laboratories must have the following personnel:
      (1) Laboratory director
      (2) Technical consultant
      (3) Clinical consultant
      (4) Testing personnel
         (a) Each person performing moderately complex testing must:
            (i) Follow laboratory procedures
            (ii) Maintain records
            (iii) Adhere to policies
            (iv) Identify problems
            (v) Document corrective actions
         (b) Testing personnel must have documented training in all areas of testing
            performed.
   b. Highly complex laboratories are required to have the same personnel as a mod-
      erately complex laboratory, but must also have:
      (1) General supervisor
      (2) Technical supervisor

4. Quality control (QC) must be performed on all moderately and highly complex test-
   ing.
a. General QC procedures are performed on:
   (1) All specialties and subspecialties as specified within the Federal Register
   (2) Procedures not found in the Federal Register, which must be conducted according to the manufacturer’s protocol that has been approved by the Food and Drug Administration

b. The laboratory must establish and follow written QC procedures for monitoring and evaluating the test process to assure accuracy and reliability of results.

c. There must be documentation of all calibration procedures.

d. A minimum of two levels of control materials are run on each day of testing.

e. All QC records must be retained for 2 years.

f. QC must be tested in a manner that provides results within the laboratory’s stated performance requirements.

g. The laboratory must have instruments, reagents, and supplies sufficient for the type and volume of tests performed.

h. There must be defined criteria for proper storage of reagents and specimens.

i. A procedure for remedial actions must be established when QC fails.

j. All reagents, solutions, medias, QC materials, and standards must be labeled.

k. Different lot numbers are not to be interchanged unless specified by the manufacturer.

5. Proficiency testing (PT) is mandatory for all moderately and highly complex tests listed on the Compiled list of clinical laboratory systems, assays, and examinations categorized by complexity, published in the Federal Register.

a. There are numerous CMS-approved PT programs available.

b. Laboratories receive between three and five challenges for each analyte that requires PT.

c. Challenges are received three times a year. The same personnel who routinely perform the test procedures must perform the PT.

d. Laboratories are required to score 80% on all analytes to be considered in compliance.

e. Blood bank specimens require a score of 100%.

6. A procedures manual must be written to include all testing methods used in the laboratory. It must be available and followed by all testing personnel.

a. The manual must include:

   (1) The principle and methodology of each test
   (2) Criteria for specimen collection, processing, and rejection (pre-analytical steps)
   (3) Instructions for microscopic examination procedures
   (4) Test procedures (analytical)
      (a) Step-by-step instructions
      (b) Result interpretation
   (5) Preparation of materials
      (a) Required solutions (e.g., reagents, controls, calibrators, stains, slides) and their location
      (b) Step-by-step preparation of all materials needed
      (c) Storage after preparation
      (d) Labeling and dating instructions
      (e) Safety precautions needed
      (f) Solutions used in preparation (e.g., deionized water, diluent)
   (6) Calibration procedures
      (a) Step-by-step instructions
      (b) Verifications of calibration results
      (c) Concentration and number of calibrators used
      (d) Calibration schedule
   (7) Control procedures
      (a) Materials used, name, lot number, level, frequency used
      (b) Preparation of materials
      (c) Instructions for testing controls
(d) Control limits
(e) Description of how and where control results are recorded
(f) Corrective actions taken when controls are not within limits

(8) Actions taken when results deviate from expected values:
(a) Recalibration
(b) Troubleshooting
(c) Repeats
(d) Dilutions

(9) Limitations
(a) Interfering substances
(b) Common sources of error

(10) Result reporting (postanalytical)
(a) Reference ranges
   (i) Range for different specimens (e.g., serum, plasma, urine)
   (ii) Demographic variables (e.g., age, sex)
(b) Panic values
   (i) Life-threatening results
   (ii) Results needing special attention (critical results)

(11) References
(a) Manufacturer’s product literature (e.g., inserts, manuals)
(b) Literature (e.g., textbooks, professional magazines)

(12) Specimen retention
(a) How long specimen is kept
(b) Where specimen is stored

(13) System for reporting patient results (postanalytical)
(a) Unacceptable results
(b) How results are reported to physician
(c) Protocol for panic and critical values

(14) Course of action for problems
(15) Specimen referral
(a) To whom or where specimen is referred
(b) How the specimen is sent
(c) How results are received

b. The manual must be approved. To obtain approval, the manual must be:
(1) Signed by the laboratory director
(2) Reviewed by all personnel using the procedure
(3) Reviewed and updated by the supervisor or director
c. The manual must be rewritten when new procedures are implemented.
d. The manual must be retained for future reference.

7. A quality assurance (QA) plan must be established and followed to monitor and evaluate the quality of tests.
   a. The program must be an ongoing process.
   b. It must evaluate all facets of the testing process.
   c. The program must extend to the interactions of other health care professionals in the ordering of tests.
   d. It must allow for actions to be taken to correct errors or problems.
   e. The program must monitor and evaluate:
      (1) Specimen requirements
      (2) Collection
      (3) Handling and processing
      (4) Corrective actions taken in QC and PT
      (5) Result reporting
      (6) Employee competence
   f. All QA activities must be documented and evaluated monthly.

8. Inspections are conducted every 2 years by the Department of Health and Human Services (HHS) or its designee.
   a. Laboratory inspectors have the right to:
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(1) Interview all employees
(2) Access all areas of a facility
(3) Observe all testing
(4) Review all information and data records
(5) Determine that the laboratory is operated in a safe manner
(6) Determine that the laboratory is performing tests only within their complexity category
(7) Evaluate any complaints from the public
b. The inspector may also:
   (1) Conduct unannounced inspections at any time during hours of operation
   (2) Ask personnel to perform testing on test samples that the inspector supplies
   (3) Re-inspect a laboratory at any time to evaluate accurate and reliable results

9. Sanctions may be imposed on laboratories that are found to be out of compliance with
one or more of the conditions for Medicare coverage.
   a. Suspension, limitations, or revocation of a certificate can be imposed.
   b. A civil suit can be brought against a laboratory to forbid testing.
   c. Imprisonment or fines can be levied against persons convicted of violations.
   d. HHS publishes annually a list of laboratories that have been sanctioned.

B. Occupational Safety and Health Administration (OSHA) regulations for Occupational
Exposure to Bloodborne Pathogens require laboratories to establish workplace safety
practices to prevent accidental exposure to etiologic agents in blood.
1. The regulations limit occupational exposure to blood and other potentially infectious
   materials that can transmit bloodborne pathogens and include but are not limited to:
   a. Hepatitis B virus (HBV)
   b. Human immunodeficiency virus (HIV)
2. The basis for all preventive measures is the practice of universal precautions. These
   are recommendations from the Centers for Disease Control and Prevention (CDC).
   a. Laboratory personnel must treat all human blood and other potentially infec-
      tious body fluids as if they were infected with HIV, HBV, and other bloodborne
      pathogens.
   b. This includes materials that are contaminated with blood.
3. A written exposure control plan must be in place that describes the procedures es-
   tablished to minimize employee exposure to bloodborne pathogens.
   a. There must be identification of job classification, tasks, and procedures in which
      there is the potential of exposure.
      (1) A determination must be made of the job types in which exposure could occur.
      (2) All tasks must be included in the job description.
   b. An explanation of personal protective equipment (PPE) available and in use
      must be included.
      (1) Appropriate protective equipment must be supplied to employees at no cost
          to them.
      (2) Equipment is considered appropriate only if it does not permit blood or
          other potentially infectious materials (PIMs) to pass through to an employee’s
          clothes, skin, mouth, or other mucous membranes.
      (3) Types of PPE are gloves, gowns, laboratory coats, face shields or masks, and,
          eye protection.
      (4) All employers are responsible for providing, maintaining, laundering, dis-
          posing of, replacing, and assuring the proper use of all PPE.
   c. Engineering controls must be in place to minimize exposure.
      (1) These are devices that isolate or remove the pathogen hazard from the work-
          place.
      (2) These devices must be made available to all employees.
      (3) Examples of engineering controls include:
          (a) Sharps containers and biosafety bags
          (b) Splash guards and self-sheathing needles
          (c) Biosafety cabinets and fume hoods
(d) Mechanical pipetting devices
(e) Handwashing facilities, eyewash stations, and showers

d. Work practices must be developed to reduce or eliminate employee exposure to blood or PIMs during the execution of their work tasks.
(1) Employees must fully understand these procedures.
(2) The procedures must be implemented, when appropriate.
(3) Work practice procedures include, but are not limited to:
   (a) Handwashing
   (b) No recapping of contaminated needles
   (c) No food or drink allowed in the laboratory
   (d) No mouth pipetting
   (e) Warning labels affixed where needed, and biohazard symbols placed appropriately
   (f) A dress code that ensures safety

e. Housekeeping procedures are necessary for maintaining a clean and sanitary work site, which is a responsibility of employers.
(1) All equipment and work surfaces must be decontaminated.
(2) Sharps containers must be puncture resistant.
(3) Containers for biohazard materials must be constructed to contain all contents and prevent leakage during handling, storage, transport, and shipping.
(4) Procedure for disposal of biohazard materials will be based on federal, state, and local regulations.
(5) All housekeeping personnel will handle contaminated materials with the use of PPE.

f. An HBV vaccination program must be established.
(1) HBV vaccine and vaccination series must be made available to all employees who are identified as being exposed to PIMs.
(2) The vaccination series will be offered within 10 days of the employee being assigned to an area of possible exposure.
(3) The vaccination series must be offered at no cost to the employee.
(4) The program must be established using the protocol outlined in the Federal Register.
(5) The program must include procedures for evaluation of the circumstances of an exposure incident to prevent further incidents.
(6) Exemptions to the HBV vaccination program are:
   (a) Employees who have already completed the HBV series
   (b) Employees whose antibody tests reveal an immunity
   (c) Employees for whom vaccination is contraindicated for medical reasons
   (7) Employees who decline the HBV series must sign the declination statement published in Addendum A of the Federal Register.
(8) All records are part of the employee’s permanent file and are confidential information.

g. Postexposure and follow-up evaluations are available at no cost to any employee who sustains an exposure.

h. The communication of hazards to employees is required by OSHA regulations.
(1) All employees must receive free biosafety training during working hours.
(2) Training sessions must be presented in a way that can be clearly understood.
(3) These sessions must be designed for the particular situation and institution involved.
(4) Generic information and signing of a statement will not satisfy the requirements.
(5) Instructors must be knowledgeable and must:
   (a) Demonstrate use and location of all safety devices
   (b) Document all training
   (c) Ensure that employees understand how to cope with hazards they may come in contact with during their employment
i. A record-keeping policy must be in place.  
(1) All documentation of training and exposure incidents must be a part of each employee’s permanent record.  
(2) All files are confidential information.  
(3) Medical records are transferred when an employee leaves.  
(4) All records are kept on file for the duration of employment plus 30 years.

C. OSHA regulations for Occupational Exposures to Hazardous Chemicals in Laboratories are published in the Federal Register.  
1. The laboratory must develop a systematic approach to significant risk determination.  
   a. The standard defines “employee” as an individual employed in a laboratory workplace who may be exposed to hazardous chemicals in the course of his or her assignments.  
   b. The standard defines “toxic or hazardous chemicals” as any substance that has the capacity to produce personal injury or illness to man through ingestion, inhalation, or absorption through any body surface.  
   c. The significant risk findings must be based on the following factors:  
      (1) Epidemiologic information relating to disease and mortality rates  
      (2) Evidence that shows significant risk for specific substances that are used in the laboratory  
      (3) General recognition that safe work practices are necessary to prevent adverse health effects  
      (4) Reported information about adverse health effects resulting from exposures to substances commonly used in the laboratory  
      (5) Relevant policy considerations  
2. Exposure monitoring of employees is required only when there is reason to believe that permissible exposure limits (PEL) or action levels for a substance are routinely exceeded.  
3. The standard has a provision for a written chemical hygiene plan (CHP) to be formulated and implemented by the employer.  
   a. Under the law, employees have the right to know any operations in their work area where hazardous chemicals are present.  
   b. The location and availability of the written hazard communication program and material safety data sheet (MSDS) are required.  
   c. A designated chemical hygiene officer (CHO) oversees the development, implementation, and administration of the CHP. The duties of the CHO include:  
      (1) Developing a standard operating procedure that is relevant to the safety and health of persons who work with hazardous chemicals (This need not be developed for each chemical. A generic approach that groups chemicals by their physical and chemical properties is acceptable.)  
      (2) Setting criteria that will be used by the laboratory to determine the need for and the implementation of measures to control and reduce employee exposures (The criteria should address engineering controls and PPE.)  
      (3) Describing the laboratory’s employee information and training program  
      (4) Explaining the laboratory’s program to provide OSHA-required medical examinations and consultations  
      (5) Designating specific operations that cannot be performed without prior approval  
      (6) Making provisions for providing additional protection for work with particularly hazardous materials, including:  
          (a) Selected carcinogens  
          (b) Reproductive toxins  
          (c) Substances with a high acute toxicity  
      (7) Labeling all chemicals to reflect the degree of hazard involved in their use  

D. OSHA’s final rule for Occupational Exposure to Formaldehyde was published on May 27, 1992.
1. The risk of exposure in the laboratory is great without proper engineering controls and good work practices.

2. A policy to maintain the permissible exposure limit (PEL) must be in place.
   a. PEL at or below 0.75 parts per million (ppm)
   b. Short-term exposure limit of 2.0 ppm per 15 minutes of exposure
   c. Action level is 0.5 ppm

3. The formaldehyde exposure control plan must include:
   a. Monitoring of employees prone to exposure
   b. Limitation of access to regulated areas to authorized persons who have been trained to recognize the hazards
   c. Establishment of identified and located engineering controls and work practices
   d. Provision of the necessary PPE and clothing at no cost to the employee
   e. A quick-drench shower and eyewash facility located within the immediate work area
   f. Visual inspections for spills as a housekeeping procedure and cleaning up spills using formaldehyde-resistant clothing
   g. Performance by a licensed physician of all medical surveillance procedures without cost to the employee
   h. Training of employees at the time of employment, annually, and whenever a new exposure to formaldehyde is introduced into the work area
   i. Environmental monitoring record keeping for 30 years

E. Guidelines from OSHA for Preventing the Transmission of Mycobacterium tuberculosis in Health Care Facilities are published in the Federal Register.

1. Each laboratory is responsible for having a policy on prevention of M. tuberculosis transmission, based on their own personnel’s exposure to tuberculosis.

2. This policy must include but is not limited to the following:
   a. Control measures must include the use of administrative measures to reduce the risk of exposure to persons who have infectious tuberculosis by developing risk assessments.
   b. Engineering controls include preventing the spread and reducing the concentrations of infectious droplets, as well as using personal respiratory tract protective equipment in areas where there is a risk of exposure. The most efficient of these is the high-efficiency particulate air (HEPA) filter.
   c. Risk assessments must be performed to identify and manage personnel who have tuberculosis. Management includes early treatment and isolation.
   d. A tuberculosis screening program for employees must be developed and must include the use of skin tests and follow-up treatment of all employees who have positive test results.
   e. All employees must be trained and educated in the use of PPE, hygiene, and handling of patients who have tuberculosis.
   f. The evaluation of tuberculosis infection control programs must include the effectiveness of the program and the outcome of exposures.

3. Three levels for preventing the transmission of tuberculosis include the following:
   a. The first level is to develop and implement policies for rapid identification. If a person is seen by a health care worker, who, from a history and examination, suspects tuberculosis, there should be a way to alert all other health care workers of the possibility. Each facility must determine its own policy for rapid identification.
   b. The second level is the use of PPE and respiratory tract protection (e.g., HEPA filter) to protect health care workers from the disease. This includes education and training programs for all employees, as well as the prompt evaluation of possible exposures to tuberculosis, including purified protein derivative skin tests and radiographs, if necessary.
   c. The third level is the coordination of activities with the local public health department. This includes the reporting of all cases, as well as continuation and completion of therapy.
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F. Health Insurance Portability and Accountability Act (HIPAA)
1. Issued by the U.S. Department of Health and Human Services, which addresses the use and disclosure of individuals’ health information called “protected health information.” HIPAA created the first national standard to protect individuals’ medical records and other personal health information.
2. The major goal of the Privacy Rule is that the “individual’s health information is properly protected while allowing the flow of health information needed to provide and promote the public’s health and well being.”
3. Applies to all health plans, health care clearinghouses, and any health care provider who transmits health information in electronic form.
4. For patients, “it means being able to make informed choices when seeking care and reimbursement for care based on how personal health information may be used.”
5. Each entity is responsible for HIPPA compliance via training and ensuring proper protocol is followed.

V. FINANCIAL MANAGEMENT

A. Budget process usually begins in advance of the implementation date and is prepared in phases dependent upon the fiscal year which consists of four phases.
1. Development of goals. Laboratory’s goals must be in line with those of the organization and must be achievable and measurable.
2. Budget assumptions. The organization provides a forecast of available money based on past earnings, cash flow, and changes in state or federal laws affecting reimbursement. Budget assumptions are based on annual test volume and revenue generated.
3. Forecast of expenses. Capital expenditure is an investment and, thus, the rate of return must be evaluated and used as a criterion for budgeting decisions.
4. Monitoring. After operational budget is made and implemented, reports are reviewed monthly for variances.

B. Employee scheduling. One of the most challenging aspects of being a supervisor/manager, which has a huge impact on the departmental budget.
1. Factors that impact scheduling.
   a. Test menu
   b. Education and experience Level of staff
   c. Number of employees needed for test volume
   d. Number of emergency areas served
   e. Turnaround time expectations (TAT)
   f. Level of test complexity

C. Health care reimbursement
1. Diagnostic related group (DRG) is a system to classify hospital cases into one of approximately 500 groups. The system was developed for Medicare as part of the prospective payment system. DRGs are assigned by a “grouper” program based on diagnoses, procedures, age, sex, and the presence of complications or comorbidities. DRGs have been used since 1983 to determine how much Medicare pays the hospital, since patients within each category are similar clinically and are expected to use the same level of hospital resources.
2. Current procedural terminology (CPT) is a listing of descriptive terms and identifying codes for reporting medical services and procedures. The purpose of CPT is to provide a uniform language that accurately describes medical, surgical, and diagnostic services, and thereby serves as an effective means for reliable nationwide communication among physicians, and other health care providers, patients, and third parties. This system of terminology is the most widely accepted medical nomenclature used to report medical procedures and services under public and private health insurance programs.
3. Medicare is a social insurance program administered by the United States government, providing health insurance coverage to people who are either age 65 and over, or who meet other special criteria.

4. Medicaid is the United States health program for individuals and families with low incomes and resources. It is an entitlement program that is jointly funded by the states and federal government, and is managed by the states. Among the groups of people served by Medicaid are eligible low-income parents, children, seniors, and people with disabilities.

5. Third-party payment is the practice of an insurer paying providers directly for services rendered to an insured, as opposed to an indemnity contract that pays the insured person for the services incurred.

VI. LABORATORY INFORMATION SYSTEMS (LIS)

A. Benefits of an LIS
   1. Reduces time-consuming paper work
   2. Stores patient data
   3. Stores QC data
   4. Provides immediate access to results
   5. Reduces the chance of an assay being run on the wrong patient when barcode is used
   6. Managerial reports (tests per month, workload units, TATs)

B. Minimum requirements for LIS
   1. Access codes or passwords per user for access to system
   2. Assign a unique accession number
   3. Provide a unique file for each assay (sample type, volume required, ranges)
   4. Record time collected, received, and unique identifier of who received it, and who released results
   5. Print bar-code labels (ISBT for immunohematology/transfusion services)
   6. Interface with instruments and other information systems

VII. INSTRUMENT SELECTION PROCESS

A. Needs assessment
   1. Characteristics of the individual laboratory
   2. Test volume (Random access vs. batch testing)
   3. Throughput (TAT for STATs)
   4. Connectivity (LIS)
   5. Test menu for present and future needs
   6. FDA approved
   7. Maintenance downtime (mean time between downtime)
   8. Sample dilution (manual vs. automatic)
   9. Ease of troubleshooting
   10. Special electrical or plumbing requirements
   11. Waste management
   12. Amount of heat generated
   13. Safety features

B. Minimum standards for quality
   1. Linearity
   2. Sensitivity
   3. Specificity
   4. Accuracy
   5. Precision
6. Stability of hardware/software (bar coding and ID features)
7. Stability and availability of reagents

C. Cost
   1. Instrument itself
   2. Reagents
   3. Contracts (service, labor, travel)
   4. Interface costs (LIS)

VIII. PROBLEM SOLVING

A. Define the problem
B. Create scenarios
C. Implement solution
D. Examine solution
E. Reflect (on problem/solution)

IX. DELIVERY OF EDUCATION UNIT

A. A goal is a general statement which describes the instructor’s purpose for a particular instructional activity.
B. Objectives are statements that describe the specific learning outcomes of an instructional activity and are written in greater detail than goals.
   1. Cognitive. Intellectual learning outcome such as the recall of information, the comprehension of that information, and the processes of analysis, synthesis, and evaluation.
   2. Affective. Emphasize values, attitudes, and interests.
   3. Psychomotor. Require neuromuscular coordination such as performing a task.
C. Learning activities allow the student to master the objectives and are comprised of lectures, laboratory session, demonstration, computer assisted instruction, internet resources, tutorials, and online courses.
D. Evaluation determines the effectiveness of the learning activity and provides feedback regarding the learner’s attainment of the objectives for the course.

X. OUTREACH PROGRAM

A. Physicians are treating patients more often on an outpatient basis; therefore, outpatient volumes can potentially reach upwards of 60% of a laboratory’s total test volume.
B. Added-value services
   1. Phlebotomy
   2. Turn around times TATs (Outpatient volumes can cause TATs to increase for inpatient testing)
   3. Supplies
   4. Courier service
   5. Reports (electronic, hand delivered, or both)
   6. Customer service staff’s (knowledge of the outreach program and its goals are critical)
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C. Know your competition
   1. SWOT analysis (strengths, weaknesses, opportunities, and threats)

D. Pricing. One of the most important aspects of an outreach program.

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