

INTEGRATED HEMATOLOGY SYSTEM

Spring, 2014

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School of Medicine
and Public Health

UNIVERSITY OF WISCONSIN-MADISON

Integrated Hematology System

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Integrated Hematology System

2014 Schedule

| Date/Time | Event | Lecturer | Reading |
|--|--|--------------------------------|---------------|
| DAY 1 Tuesday, Feb. 4: 8:00-8:40 am 8:40-9:40 am 9:40-10:10 am 10:20-12:00 Noon | Hematopoiesis Introduction & Patient Interview Lecture: Hematopoiesis Pathology Discussion Small Groups | Erik Ranheim Erik Ranheim | Chap 1 |
| DAY 2 Wednesday, Feb. 5: 8:00-9:50 am | Anemia Lecture: Anemia & Iron w/10 min break | Eliot Williams | Chap 2 |
| DAY 3 Thursday, Feb. 6: 8:00-8:30 am 8:30-9:00 am 9:10-9:40 am 9:50-12 noon | Anemia Patient Interview Lecture: B-12 & Folate Deficiency Pathology Discussion Small Groups & Quiz | Eliot Williams Erik Ranheim | Chap 3 |
| DAY 4 Friday, Feb. 7: 8:00-8:30 am 8:30-9:50 am | Anemia Patient Interview Lecture: Overview of Hemolysis, Membrane & Enzyme Defects | Elizabeth Silverman | Chap 4 |
| Quiz 1: Day 1-4 material – Begins February 7 | | | |
| DAY 5 Monday, Feb. 10: 8:00-8:30 am 8:30-9:25 am 9:30-9:50 am 10:00-12 noon | Anemia Patient Interview Lecture: Globin Disorders Pathology Discussion Small Groups & Quiz | Carol Diamond Erik Ranheim | Chap 5 |
| DAY 6 Tuesday, Feb. 11: 8:00-8:50 am 9:00 am-12 noon | Anemia Lecture: Transfusion Medicine Small Groups (wet lab) and Quiz | Nick Rose | Chap 6 |

| Date/Time | Event | Lecturer | Reading |
|--|--|---|-----------------------------------|
| DAY 7 Wednesday, Feb. 12: 8:00-8:30 am 8:30-9:15 am 9:30-9:50 am 10:00-12 noon | Anemia Patient Interview Lecture: Immune Hemolysis Pathology Discussion Small Groups & Quiz | Eliot Williams Erik Ranheim | Chap 7 |
| QUIZ 2: Days 5-7 material – Begins February 12 | | | |
| DAY 8 Thursday, Feb. 13: 8:00-8:30 am 8:30-9:25 am 9:30-10:10 am 10:20-12 noon | Myeloid Neoplasia Patient Interview Lecture: Myeloid Neoplasms Pathology Discussion Small Groups & Quiz | Mark Juckett Erik Ranheim | Chap 8 |
| DAY 9 Friday, Feb. 14: 8:00-8:30 am 8:30-9:25 am 9:30-10:10 am 9:30-12:00 Noon | Lymphoproliferative Disorders Patient Interview Lecture: Lymphoma Pathology Discussion Small Groups & Quiz | Brad Kahl Erik Ranheim | Chap 9 |
| DAY 10 Monday, Feb. 17: 8:00-8:55 am 9:00-9:55 am 10:00-10:10 am 10:20-11:30 am | Plasma Cell Disorders Lecture: Plasma Cell Disorders Lecture: Molecular Basis of Hematologic Malignancy Pathology Discussion Small Groups & Quiz | Fotis Asimakopoulos Erik Ranheim Erik Ranheim | Chap 10 Chap 10B |
| QUIZ 3: Days 8-10 material – Begins February 17 | | | |
| DAY 11 Tuesday, Feb. 18: 8:00-8:30 am 8:40-9:30 am 9:35-10:25 am 10:30-10:50 am | Hemostasis Small Groups (Quiz only) Lecture: Hemostasis Lecture: Antithrombotic drugs Lecture: Hemostasis testing | Brad Schwartz John Sheehan Eliot Williams | Chap 11 |
| DAY 12 Wednesday, Feb. 19: 8:00-8:30 am 8:30-9:20 am 9:30-11:00 Noon | Bleeding Disorders Patient Interview Lecture: Bleeding Disorders Small Groups | Eliot Williams . | Chap 12 |

| Date/Time | Event | Lecturer | Reading |
|---|--|--------------|----------------|
| DAY 13 Thursday, Feb. 20: 8:00-8:30 am 8:30-9:20 am 9:30-11:00 am | Thrombosis Patient Interview Lecture: Thrombosis Small Groups & Quiz | John Sheehan | Chap 13 |
| DAY 14 Friday, Feb. 21: 8:00-8:30 am 8:40-9:55 am | Wrap-up Small Groups (Quiz only) Review Session | | |
| QUIZ 4: Days 11-13 material – Begins February 21 | | | |
| Monday, Feb. 24 TBA | FINAL EXAM | | |

Integrated Hematology Faculty Listing

| <u>INSTRUCTORS</u> | <u>OFFICE ADDRESS</u> | <u>OFFICE PHONE</u> | <u>E-MAIL ADDRESS</u> |
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| Fotis Asimakopoulos, MD (Assistant Professor of Medicine) | 4031 WIMR | 265-4363 | fasimako@medicine.wisc.edu |
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CHAPTER 1

INTRODUCTION TO HEMATOLOGY

Key Concepts:

- Composition of blood
- Morphology and function of normal blood cells
- Biology and morphologic correlates of hematopoiesis
- Clinical significance of high or low levels of specific blood cells
- Consequences of bone marrow failure.
- Clinical use of hematopoietic growth factors

Learning Objectives:

1. Be able to identify the cells found in normal blood, and describe the functions of each cell.
2. Describe the properties of stem cells and their role in hematopoiesis.
3. Describe how hematopoiesis is regulated by cytokines such as erythropoietin and G-CSF to ensure an adequate supply of blood cells.
4. Be able to recognize the precursors of red cells and neutrophils at various stages of development.
5. Describe the consequences (signs and symptoms) of having too many or too few of the major cell types in peripheral blood.
6. Describe the pathophysiology and the main clinical and pathologic features of aplastic anemia.
7. Describe the clinical uses of recombinant erythropoietin and granulocyte colony-stimulating factor.

Blood is beautiful. It is symbolic of life, courage, and sacrifice. Englishmen swear by it. The Romans loved to shed it in the arena. The ancient Hebrews made it sacred in their sacrifices. Its color fascinates flag-makers and artists. Students since Ehrlich have been delighted with the shapes and colors of red cells and leukocytes. The intellectual beauty of blood is apparent to the physiologist and biochemist studying the orchestration of cells, gases, substrates, stimulators, and inhibitors.

I. Blood and Its Constituents

Hematology is a discipline concerned with the production, function, and disorders of blood cells and blood proteins. We are familiar with blood from the time of our first skinned knee, but what is it really made of?

Blood is a liquid consisting of plasma (water, electrolytes, nutrients, waste products, and many soluble proteins) in which red cells, platelets and a variety of white cells are suspended. Its volume is about 70 mL/kg or about 5L total in an average size adult. 40-50% of blood volume is occupied by red cells.

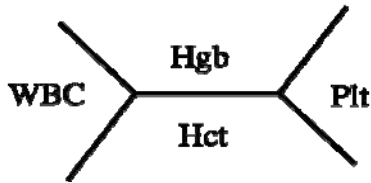
Some of the important proteins in the blood are:

- Albumin, which contributes a large portion of the oncotic pressure of plasma.
- Immunoglobulins (antibodies), which combat infection
- A number of proteins involved in the clotting cascade.

The cellular components of blood include:

- Red blood cells (RBC, about $5 \times 10^6 / \mu\text{L}$), which carry oxygen to tissues
- Platelets (about 250,000 / μL), which facilitate clotting.
- White blood cells (WBC, about 5,000 / μL), which fight infection. WBC can be further subdivided into neutrophils, lymphocytes (T, B, and natural killer [NK] cells), monocytes, eosinophils, and basophils (listed in order of typical frequency).

One of the most common tests ordered by all medical specialties is the **complete blood count, or CBC**. The clinical laboratory uses an analyzer that functions both as a spectrophotometer and a flow cytometer. This instrument can determine the concentration (count) of RBC, platelets and WBC, the hemoglobin (Hgb) concentration, and the mean size of the red cells (MCV). The flow cytometer can accurately distinguish and count the various types of WBC, which it reports as a “differential”. The hematocrit (Hct), which represents the percentage of blood volume occupied by red cells, is calculated from the RBC count and the MCV. In conferences and charts, you will often see this reported in a shorthand fashion like this:



As we will repeatedly emphasize in this text and this course, **the CBC results can tell you a great deal about potential disease processes affecting a patient.**

The normal ranges for the values of the CBC are listed in Tables 1.1 and 1.2.

Table 1.1
NORMAL VALUES FOR RBC AT SELECTED AGES

| Age | RBCx10 ⁶ (per μl) | Hgb (g/dl) | Hct (%) | MCV (fl) |
|----------------|---|---------------|---------|-------------|
| 1 day | 5.1 | 19.5 | 54.0 | 106 |
| →60 day | 4.7 | 14.0 | 42.0 | 90 |
| 6-12 m. | 4.6 | 11.8 | 35.5 | 77 |
| 1-2 yr. | 4.5 | 11.4 | 35.0 | 78 |
| 3-10 yr. | 4.6 | 12.6 | 37.0 | 80 |
| <u>Adults:</u> | | | | |
| Females | 4.5±0.7 | 13.7±2 | 41.0±6 | 87±05 |
| Males | 5.1±0.7 | 15.5±2 | 46.0±6 | 87±05 |

- a) Note the higher values in the newborn. This is due to the predominance of HgbF (fetal), which is more efficient at extracting oxygen from the placenta but less efficient in delivering oxygen to the tissues (left shifted O₂ dissociation curve, see below).
- b) The differences between adult males and females are due to testosterone, which increases erythropoietin production. In aged men, testosterone levels fall and the disparity between men and women decreases.
- c) The mean cell volume (MCV) is measured by the flow cytometer in most laboratories. It can be a helpful clue as to the cause of low Hgb levels, as we will discuss in later chapters.

Table 1.2
NORMAL ADULT PERIPHERAL BLOOD WHITE CELL VALUES

| | <u>Absolute</u> <u>Mean/μl</u> | <u>Absolute Count</u> <u>Range</u> | <u>Average</u> <u>Percentages</u> |
|-----------------------|--|---------------------------------------|--------------------------------------|
| TOTAL WBC | 7,200 | 4,400 – 11,500 | (100) |
| segmented neutrophils | 3,800 | 1,600 – 6,700 | 50 |
| lymphocytes | 2,600 | 1,300 – 4,200 | 35 |
| monocytes | 410 | 110 – 810 | 6 |
| band neutrophils | 370 | 50 – 1,400 | 6 |
| eosinophils | 200 | 0 – 575 | 3 |
| basophils | 33 | 0 – 160 | 0.6 |

Note: the “band” neutrophil is an immature neutrophil released “early” from the bone marrow, e.g., in response to a bacterial infection

The values of cell counts, hemoglobin, and hematocrit are in part dependent on the plasma volume. We usually assume that the plasma volume is normal (about 40 ml/kg). If the plasma volume is reduced, as in dehydration, the cell counts, hemoglobin, and hematocrit will be falsely elevated. On the other hand, if the plasma volume is increased, they will be falsely low. **When red cells and plasma are lost simultaneously in acute bleeding, the hematocrit will initially be normal but the total blood volume will be reduced. The WBC differential reports each cell type as a percentage of the total WBC. The absolute number of cells in each class per microliter of blood is obtained by multiplying the percentages by the white blood cell count. It is very important to think in absolute values rather than percentages.**

II. The Cells of the Peripheral Blood

A. Red Blood Cells (Erythrocytes)

The RBC is a masterpiece of design, but also incredibly simple – essentially a bag of hemoglobin. Its thin flexible membrane, in the unusual shape of a **biconcave disc**, is ideal for gas transport (high surface area: volume ratio). The red cell is so **pliable** that it can pass through spaces half its diameter, yet its membrane is **rugged** enough to remain intact for four months and hundreds of miles. Like a good automobile tire, it is self-sealing. It carries no nucleus to impede gas exchange or add a metabolic burden. It requires only a relative handful of nonrenewable enzymes to maintain its membrane

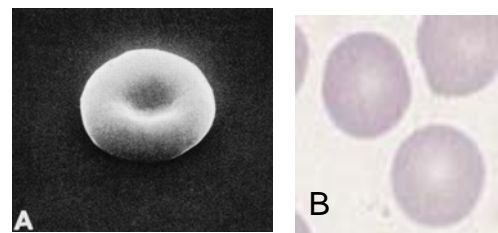
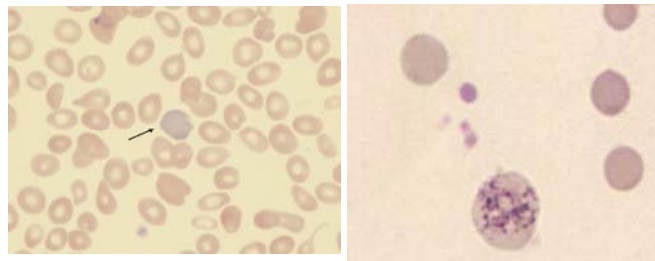


Figure 1.1. A) RBC as seen by scanning electron microscopy (S. Schrier, ASH Image Bank). B) RBC seen on Wright's stain of peripheral blood smear.

integrity and repair oxidative damage to its hemoglobin molecules. This enzymatic machinery also regulates its oxygen binding capacity in different environments. Finally, at the end of its 4-month life, the RBC is eaten by splenic macrophages and almost completely **recycled**.

When Wright-stained peripheral blood smears are examined under the microscope, RBC generally appear as in Figure 1.1B, with the thin center of the biconcave disc appearing pale or white and the thicker edges appearing red-pink. In Wright or Wright-Geimsa staining which is typically used in smear preparations in hematology, proteins stain red and DNA/RNA stain blue/purple. In RBC, the pale-staining area should account for no more than one third the diameter of the RBC (**normochromic**). If it exceeds one-third of the diameter, the RBC is said to be **hypochromic**, suggesting a paucity of hemoglobin. Normal red blood cells are about 8 μm in diameter (85-95 fL in volume), while hypochromic cells (less full of Hgb) are often smaller.

Young red blood cells just emerging from the bone marrow still contain residual RNA, which stains blue on Wright stain, causing these somewhat larger, rounder cells to look like purple spheres without a clear central pale zone (Figure 1.2. left). These **reticulocytes** mature over the



course of one to two days into the typical biconcave disc cell as their RNA degrades. Normally, about 1% of RBC are reticulocytes (about 50,000/ μL), which makes sense - if their life span is 120 days, then about 1% of the cells “die” every day and need to be replaced. If red cells are being destroyed or lost at a faster rate

Figure 1.2. Reticulocytes are young RBC that still contain RNA. On Wright stain (left) they appear large and purple. If methylene blue staining is used (right) the ribosomes in the reticulocyte become visible. In the course, and in the clinical lab, we typically will do only a Wright stain of peripheral blood. (photo credit http://www.healthsystem.virginia.edu/internet/hematology/Hes_sEDD/BenignHematologicDisorders/normal-).

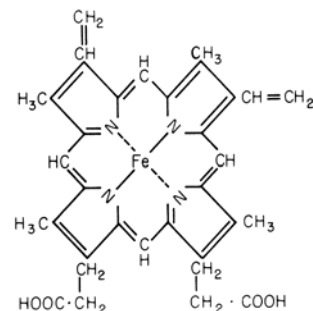
due to hemolysis or bleeding, reticulocyte numbers should rise. An increase in range of sizes of RBC is referred to as **anisocytosis**, while an increase in different shaped RBC is called **poikilocytosis**. The significance of different RBC shapes (e.g., spherocytes, tear-drop cells, schistocytes, etc.) will be discussed in the anemia sections of the text.

RBC BIOLOGY

We will focus in this introduction on three main problems faced by the RBC and how its biologic make up solves these problems.

1. The RBC must pick up oxygen in the lungs and leave it in peripheral tissues where it is needed.
2. The RBC, having no nucleus, cannot make new proteins or heme molecules over its 4 month life span, so must have mechanisms in place to repair damage caused by oxidants, drugs, etc.
3. An 8 μm wide cell must fit through 3 μm wide capillaries, over and over again.

Problem 1 – Oxygen



The RBC is packed with hemoglobin (Hgb), a rather remarkable tetrameric protein consisting of two α chains and two β chains (in Hgb A, which comprises the great majority of hemoglobin in adults). Each chain contains a heme molecule, a cyclic tetrapyrrole with a Fe^{++} ion at its center where O_2 is bound (Figure 1.3). When one heme molecule binds to O_2 , a conformational change occurs in the globin chains to make further O_2 binding to other chains more efficient (Figure 1.4). The saturation curve for O_2 binding to Hgb is sigmoidal. There is nearly 100% saturation at the partial pressures of O_2 seen in arterial blood, but the saturation falls sharply at the O_2 pressures encountered in venous blood (Figure 1.5). In this way, O_2 is loaded onto Hgb in the pulmonary capillaries and offloaded to the tissues in the peripheral capillary beds. This process is modulated by shifts in the saturation curve due to surrounding pH, $[\text{CO}_2]$, and changes in the level of the regulatory molecule 2,3-DPG. With increasing acidity and $[\text{CO}_2]$, as found in exercising muscle, the curve shifts to the right, decreasing Hgb's affinity for O_2 and promoting its release in O_2 -starved tissues. Increasing 2, 3-DPG also decreases the affinity for O_2 . The production of 2, 3-DPG by the RBC is increased by hypoxemia in states such as chronic lung disease, congestive heart failure, high altitude, and anemia. In the placenta, DPG binds much more avidly to adult Hgb (Hgb A) than to fetal Hgb (Hgb F). This decreases oxygen affinity of adult Hgb relative to fetal Hgb and facilitates O_2 transfer from mother to fetus.

Figure 1.3. Heme molecule ready for binding to a globin chain

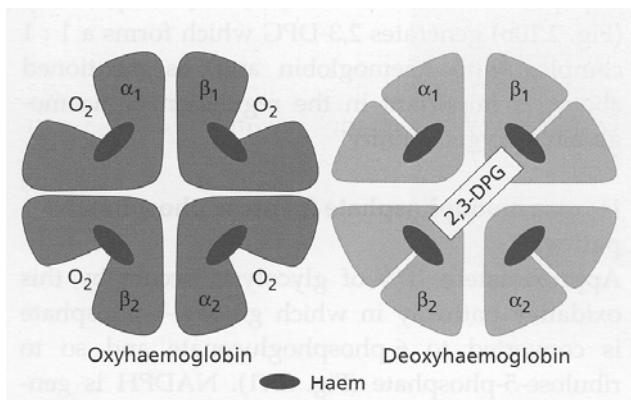


Figure 1.4. Binding of O_2 to the heme (or haem as the British say) causes conformational changes in the globin chains. Binding of 2,3-DPG favors conformations where O_2 is released. (From Hoffbrand et al. *Essential Haematology*, 5th Ed.)

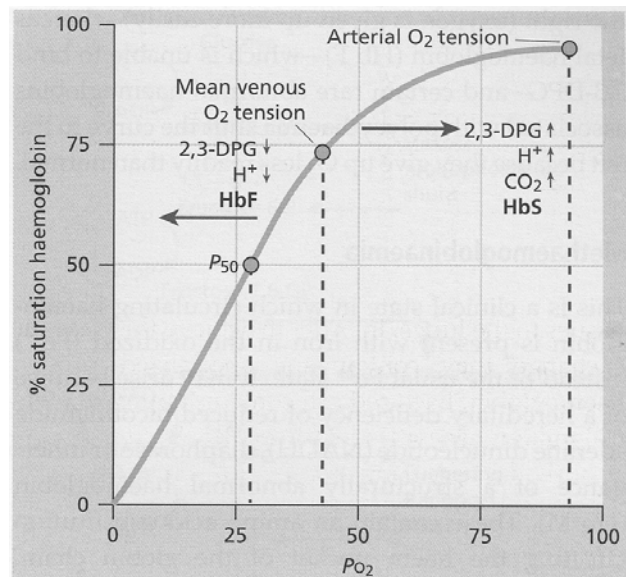


Figure 1.5. Oxygen saturation of Hgb A at different PO_2 levels. Increased acidity, CO_2 , and DPG shift the curve to the right, meaning lower affinity and saturation at a given PO_2 . (From Hoffbrand et al. *Essential Haematology*, 5th Ed.)

Problem 2 – No Nucleus

Since the RBC really only has one job—carrying oxygen through tight spaces—the lack of a nucleus seems like a good thing. However, a variety of chemicals and naturally formed oxygen radicals can damage now irreplaceable RBC proteins and oxidize the ferrous iron (Fe^{++}) in heme to the ferric (Fe^{+++}) state. Hemoglobin containing only Fe^{+++} does not bind oxygen at all, while partially oxidized Hgb containing some ferric and some ferrous iron (**methemoglobin**) has a left-shifted saturation curve, and thus is unwilling to give up its O_2 in peripheral tissues. The RBC also lacks mitochondria, and must use the Embden-Meyerhof glycolytic pathway, which converts glucose to lactate, for its energy needs (primarily running ion transporters to counteract the enormous oncotic pressure of all that hemoglobin). This pathway fulfills a number of other needs for the erythrocyte (Figure 1.6).

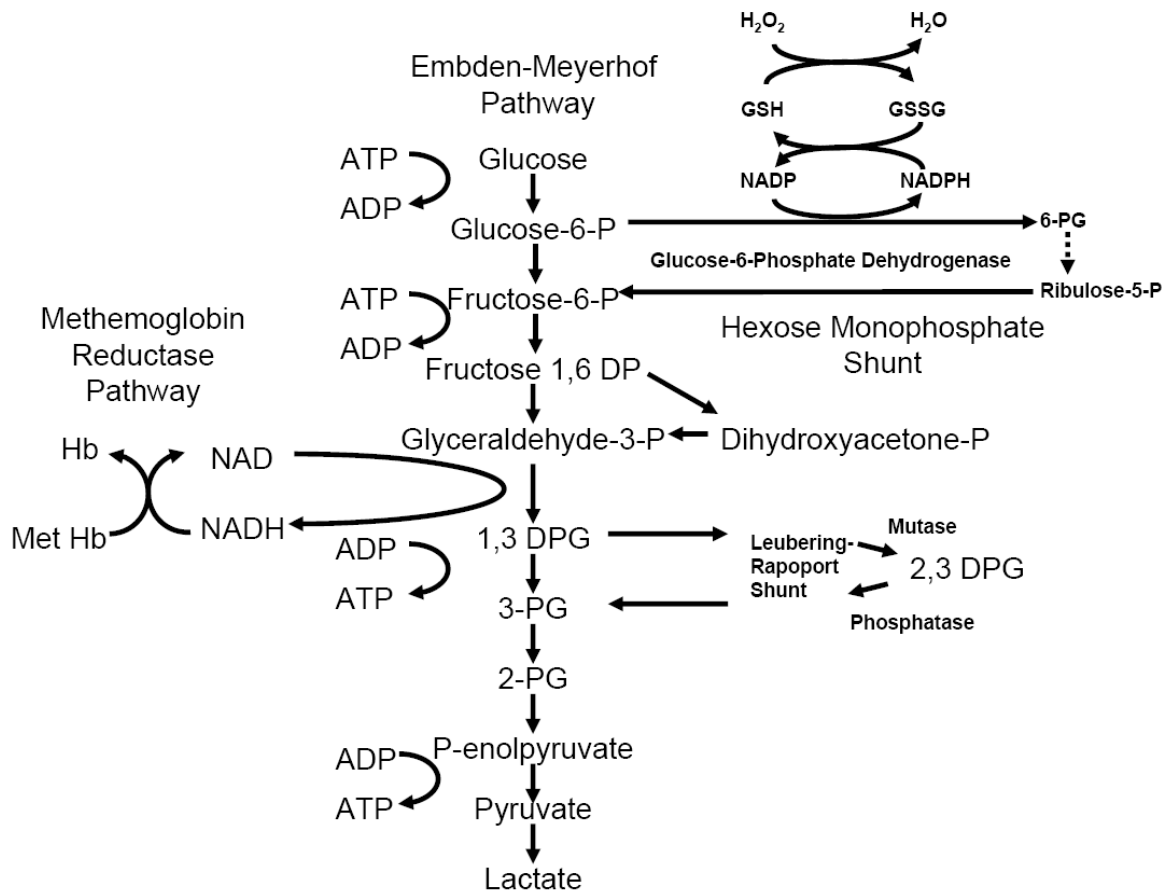


Figure 1.6. Metabolic pathways in the RBC

- First, it produces NADH, which the cell uses to convert Fe^{+++} (methemoglobin) back to its Fe^{++} state.
- Second, the RBC produces 2, 3-DPG by employing the Leubering-Rapaport shunt.
- Finally, about 10% of glucose metabolism streams through the hexose monophosphate shunt, regenerating the NADPH used by the glutathione pathway to reduce oxygen radicals that would otherwise damage cellular membranes and irreplaceable proteins.

Problem 3 – A tight squeeze

Despite its inability to replace lost proteins or lipids, a RBC needs to be durable enough to withstand hundreds of miles of bumpy travel over a lifetime, yet flexible enough to squeeze through capillary beds that measure less than half its own diameter. The RBC membrane is an engineering masterpiece, containing only 20% phospholipid, with 50% being protein, 10% carbohydrate, and 20% cholesterol, allowing for remarkable membrane flexibility. Several of the membrane proteins comprise a net-like cytoskeleton that underlies the membrane, providing flexibility and stability (Figure 1.7). Mutations of these structural proteins can lead to abnormalities in RBC size, shape, and flexibility. Some of these are typically asymptomatic, such as hereditary elliptocytosis (ovalocytosis) (Figure 1.8), while others cause hemolysis and anemia, such as hereditary spherocytosis, or a much rarer (and more severe) condition called hereditary pyropoikilocytosis. Hereditary spherocytosis and the RBC membrane will be discussed in detail in chapter 4.

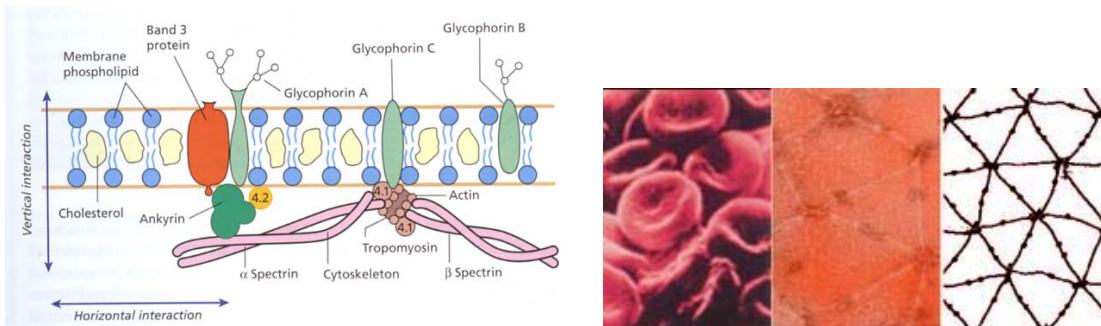
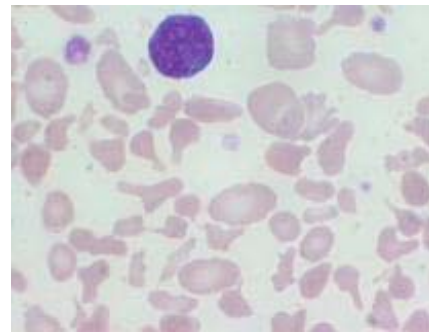
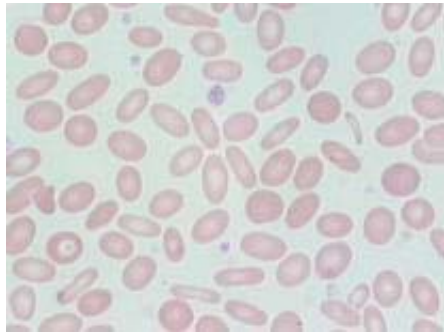


Figure 1.7. The RBC membrane and some its more critical protein components (left, from Hoffbrand et al. *Essential Haematology*, 5th Ed.). The spectrin chains form a net-like protein scaffold beneath the cell membrane that is flexible yet sturdy (right, from C Vera, R Skelton, F Bossens, and LA Sung. 2005. *Annals of Biomedical Engineering*. 33 (10), pp 1387–1404.)



FYI

Figure 1.8. Peripheral blood smears from patients with hereditary ovalocytosis (left) and a rare condition called hereditary pyropoikilocytosis (right), resulting from heterozygosity and homozygosity, respectively, for mutations in the spectrin gene (Images from the ASH Image Bank).

B. The Platelet

Platelets are the second most abundant cell in peripheral blood. Technically they are not cells at all, but rather membrane-bound fragments of cytoplasm from a large precursor in the bone marrow, the **megakaryocyte**. The platelet contains a complex internal structure that includes structural filaments and specialized secretory granules. **It functions to plug holes in blood vessels.** When a vessel wall is injured and surface endothelium is disturbed, platelets adhere to the injury site and release chemical mediators that attract other platelets to form a gluey mass.

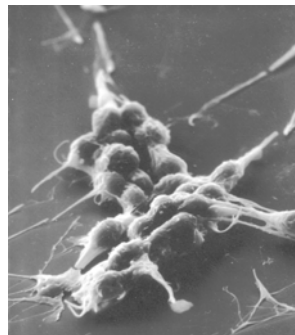
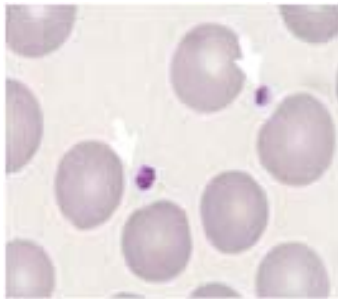


Figure 1.9. Platelets in Wright-stained smear (left, arrows) and clustered on an endothelial cell by EM (Thomas Caceci, ASH Image Bank).

This blood vessel "glue" is not tough enough to keep the blood from leaking out indefinitely, but the mass serves as an active site on which long fibrin strands form. Fibrin binds the platelets down, much like wire mesh holds the cork in a champagne bottle, until healing of the vessel wound is organized. On the peripheral smear, platelets appear as irregularly shaped cells with azurophilic (purple-red) granules. These cells are about one-fourth the size of RBC. Platelets live in circulation for about one week (8-10 days) under normal conditions.

C. The Neutrophil and Other Granulocytes

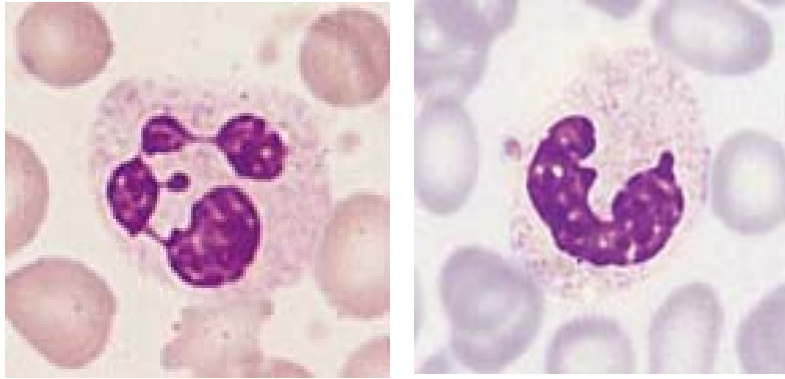


Figure 1.10. Neutrophil (left) and Band form (right) as seen on Wright stained peripheral blood film.

The neutrophil (also called polymorphonuclear leukocyte, or PMN) is usually the most abundant of the WBC in the peripheral blood. It is an ameba-like phagocyte, loaded with a variety of potent enzymes. The first white cell on the scene of inflammation, it lives a relatively short life of at most 5-7 days after leaving the bone marrow. After ingesting and digesting bacteria, the neutrophil self-destructs from the effects of its released enzymes

and oxidants. On the peripheral smear (Figure 1.10), neutrophils are easily identified by their segmented nucleus and indistinct pink granules in their cytoplasm. They are 15-20 μm in diameter, about three times the size of the RBC. Immature neutrophils in which the nucleus has not completely segmented are referred to as “bands”; these increase in number during bacterial infections because they leave the bone marrow early. About 50% of neutrophils at any given time are loosely attached to vessel walls (marginated), but can be released into circulation with stimuli such as stress or exercise.

Eosinophils and basophils, the other WBC with large numbers of prominent granules (Figure 1.11), normally make up a small fraction of WBC. Increased eosinophils can be seen in association with asthma or allergic reactions, helminth infections, or in association with certain malignancies or drug reactions. An increase in basophils suggests the presence of a primary bone marrow proliferative disorder (for example, chronic myelogenous leukemia, discussed in chapter 8).

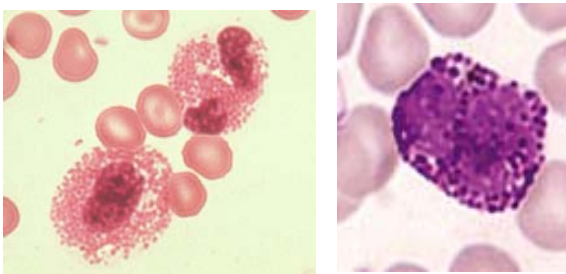


Figure 1.11. Eosinophils (left) typically show bi-lobed nuclei and large, monotonous orange-red granules. Basophils (right) have segmented nuclei with large, chunky purple granules that can obscure the nucleus.

D. Lymphocytes

Lymphocytes are typically slightly less numerous than neutrophils in the peripheral blood, except in young children. These small (about 10 μm , slightly bigger than RBC), mononuclear cells have relatively scant, blue-staining cytoplasm and round nuclei with dense, sometimes clumpy chromatin (Figure 1.12). It is not possible to distinguish T cells from B cells by morphology. Both can become larger, with increased cytoplasm, upon activation. Large granular lymphocytes, a mixture of NK (natural killer) cells and cytotoxic (CD8+) T cells, make up a special class of lymphocytes. These have increased cytoplasm containing a few purple-red granules. They are sometimes referred to as “atypical lymphocytes”. Lymphocytes can be quite long-lived, so lymphocyte counts do not necessarily tell us much about the patient’s current bone marrow output. Increased lymphocytes may indicate a lymphoproliferative neoplasm but also occur in viral and bacterial infections, most notably in infectious mononucleosis (Epstein-Barr virus) and whooping cough (*Bordetella pertussis*).

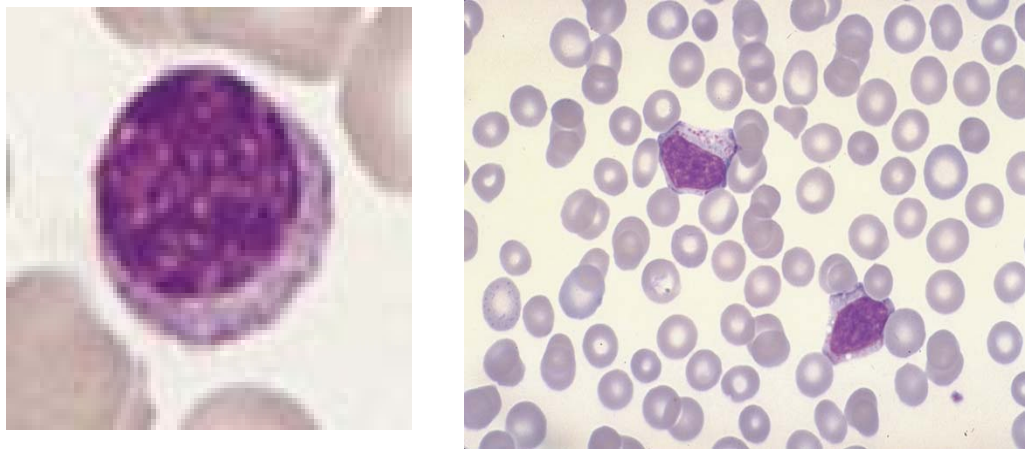


Figure 1.12. Normal lymphocyte (left) and large granular (“atypical”) lymphocytes (right).

E. Monocytes

The peripheral blood monocyte is a precursor to tissue macrophages. There are normally 200-1000 of these cells per μL . Initially, students have difficulty discriminating between activated or large granular lymphocytes and monocytes on the peripheral smear. Compared with the lymphocyte, the monocyte has more cytoplasm, which tends to be grey rather than blue, and is larger than most lymphocytes, with a bean-shaped or folded nucleus and less dense chromatin. Monocyte numbers increase in some myeloproliferative disorders and in some infections such as tuberculosis.

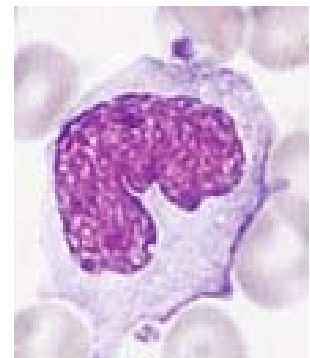


Figure 1.13. A peripheral blood monocyte with indented nucleus and abundant cytoplasm.

III. The Bone Marrow and Blood Cell Development

All of the cells in peripheral blood originate in the bone marrow in the adult human. In embryonic life, phases of hematopoiesis occur in the yolk sac, liver, and spleen, with the bone marrow becoming dominant by the time of birth (Figure 1.14)

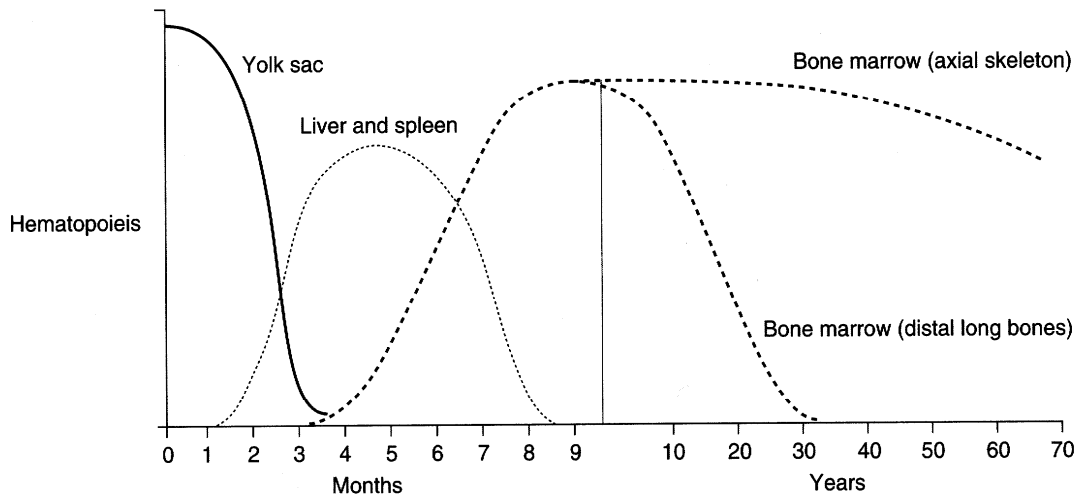


Figure 1.14. Sites of hematopoietic cell maturation at various ages (from Hoffbrand et al. *Essential Haematology*, 5th Ed.).

When it is necessary to evaluate the bone marrow microscopically, a smear of a liquid aspirate of bone marrow containing marrow cells, blood, and small spicules of bone is prepared and stained with Wright stain. In addition, a core of bone marrow is fixed, sectioned, and stained in a manner similar to other tissue biopsies. Figure 1.15 shows a typical section of a bone marrow biopsy from an adult. Note the variety of cell shapes and sizes, with precursors of neutrophils, RBC, and platelets being most prominent. The white spaces represent adipocytes (fat cells), which make up a variable proportion of marrow space that increases with advancing age. The percent of marrow space taken up by hematopoietic cells in normal circumstances is roughly 100 minus the age in years (this has a high standard deviation, however, and 100-year-old people do not have 0% cellularity). The biopsy section provides useful information about marrow cellularity and architecture, but it is difficult to precisely distinguish and quantify the lineage of the various cells (except the large megakaryocyte platelet precursors). This is typically done on the aspirate smear, as we will see below. **About 50% of marrow cells are neutrophils and their precursors, 25% erythroid precursors, and the remainder lymphocytes, monocytes, plasma cells, and others.** The ratio of myeloid (for this purpose,

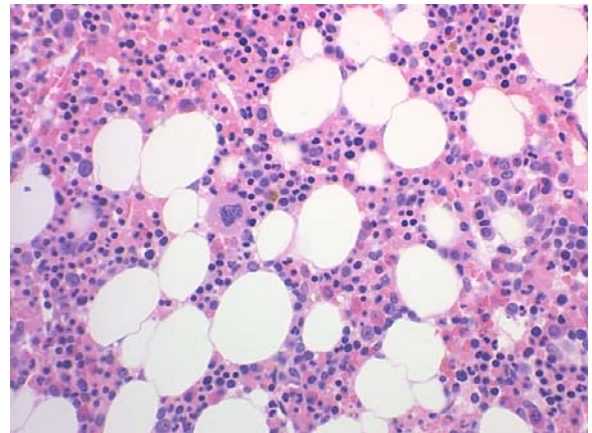


Figure 1.15. Bone marrow biopsy from a normal 40-year-old man with approximately 60% cellularity and 40% adipose tissue.

granulocyte and monocyte precursors) to erythroid precursors (called the G:E or M:E ratio) is typically about 2-3:1.

A. Hematopoiesis and Stem Cells

All blood cells originate from a collection of long term hematopoietic stem cells (LT-HSC) that are defined by the properties of:

1. **Pluripotency (the ability to produce all blood cells)**
2. **Self-renewal capacity (the ability to make copies of themselves that retain stem cell properties) for the life of the animal.**

The progeny of HSC undergo a number of cell divisions and differentiation to precursor cells with progressively more limited lineage capacities, eventually giving rise to a large cohort of mature blood cells. Except for lymphocytes and perhaps monocytes, these mature cells are post-mitotic (no longer able to divide) (Figure 1.16). Flow cytometry-based methods are able to separate these precursors by virtue of their surface protein phenotype (from the work of I. Weissman, I. Lemischka, S. Morrison, and others), and current nomenclature reflects their roles as specific precursors. For example, CLP refers to a common lymphoid progenitor that

Adult Hematopoietic Cell Ontogeny

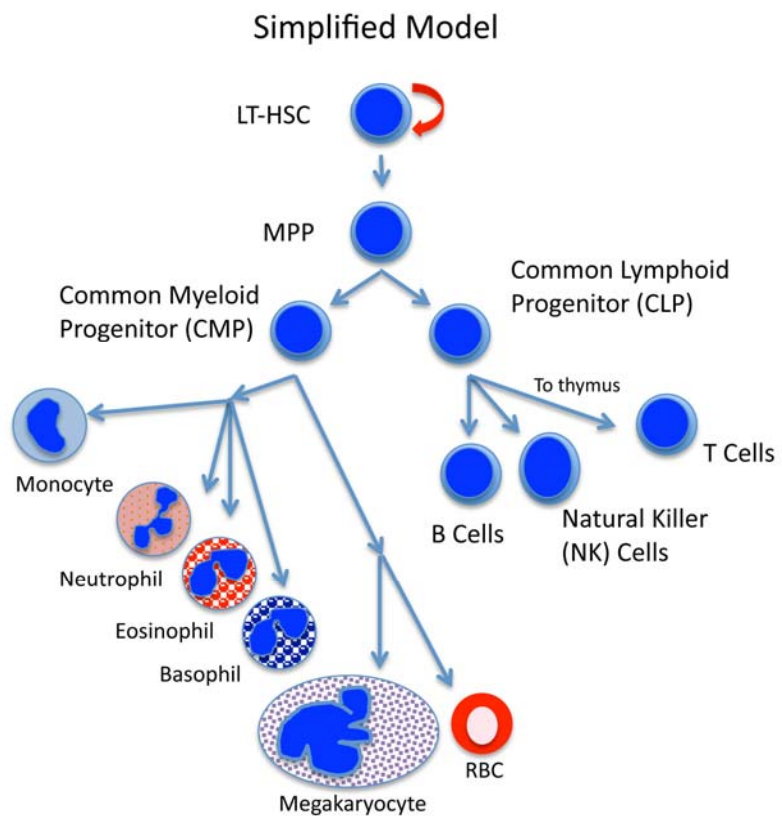


Figure 1.16. Hematopoietic ontogeny from long term hematopoietic stem cells (LT-HSC) through progenitors that do not have indefinite self-renewal capacity including the multipotent progenitor (MPP), lymphoid restricted common lymphoid progenitor (CLP), myeloid committed common myeloid progenitor (CMP). This is a simplified view of hematopoiesis as we now understand it; in mice, cells have been identified that can give rise to B cells and monocytes, for example, so this simplistic diagram is under constant revision. For our purposes, the point is that these progenitors that derive from the HSC and MPP undergo progressive restriction in their potential cellular outcomes until we have a cell committed, for example, to neutrophil differentiation. This cell will give rise to numerous promyelocytes which will in turn each proliferate and differentiate to numerous myelocytes. Only late in the process (band) will proliferation stop and the cell concentrate only on differentiating and maturing

can give rise to B cells, T cells, and NK cells, but not myeloid lineage cells. The committed

progenitor cells identified in Figure 1.16 (CMP, CLP, etc.) are rare cells that are not readily distinguished by their morphology (they appear as a range between lymphocytes and more immature-looking cells, blasts). **The bulk of the bone marrow is made up of identifiable precursors to neutrophils and RBC as described in more detail below.**

B. The Maturation of Myeloid and Erythroid Cells

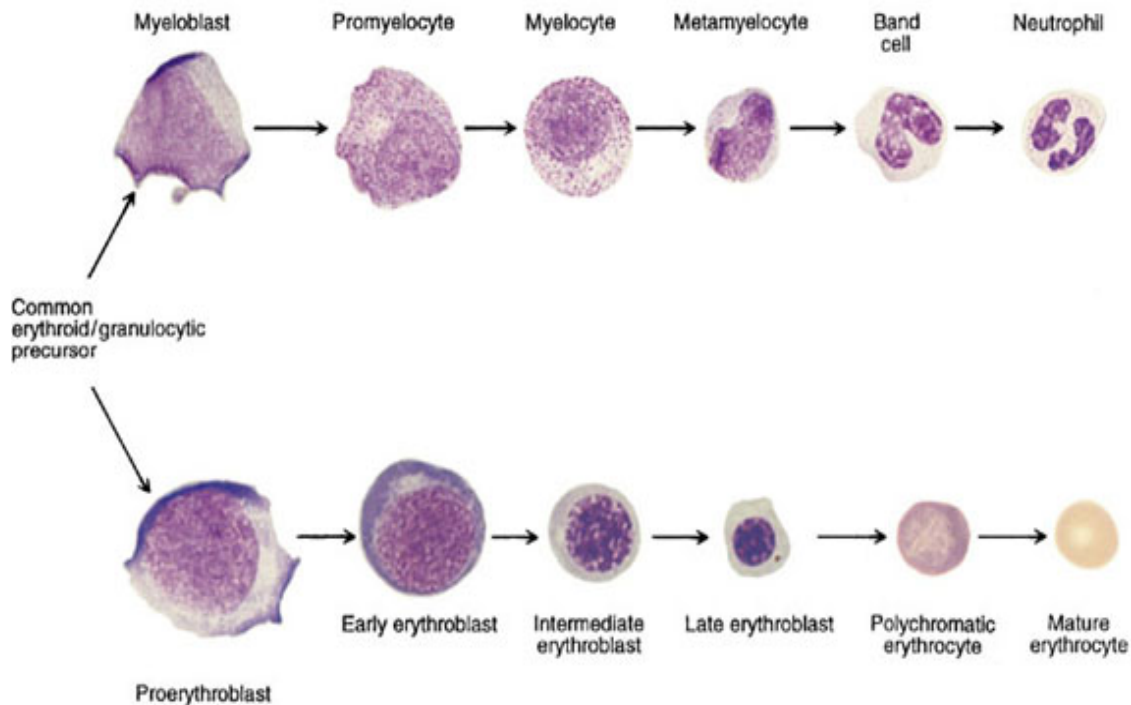


Figure 1.17. Steps in myeloid (top) and erythroid (bottom) maturation.

The maturation of mature myeloid cells (and here we are talking mainly about neutrophils) proceeds in a well-defined morphologic continuum from the myeloblast, a cell committed to neutrophil fate and a daughter of the GMP cell of Figure 1.16, through mature neutrophil (Figure 1.17, top row). We somewhat arbitrarily apply names to various stages in this maturation sequence. When performing a **differential count** on a bone marrow aspirate, cells at each of these stages are counted separately.

Morphologically, a **myeloblast** is a cell with high nuclear:cytoplasmic (N:C) ratio, delicate, open chromatin, and often containing a nucleolus (Figure 1.17). The cytoplasm is typically pale blue and may contain a few pink-purple granules. It is often difficult to tell if a blast cell is committed to myeloid or other fates without doing flow cytometric or histochemical analysis.

The next stage of maturation is the **promyelocyte**. This cell is often slightly larger than a blast, with slightly more cytoplasm and distinctive reddish granules that often are distributed to one side of the cell (Figure 1.18). The nucleus still has an immature appearance and in many cells a perinuclear clear zone representing the Golgi apparatus is apparent.

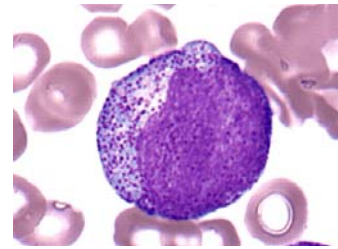


Figure 1.18. Promyelocyte

As the cell matures further, its overall size declines as does its N:C ratio. The **myelocyte** (Figure 1.17) has a round/oval nucleus and its granules are less distinct and more orange/pink than those of the promyelocyte. After this stage, the cells are post-mitotic, and as the nucleus indents and elongates we have **metamyelocytes** and **bands**, finally segmenting into lobes in the mature neutrophil.

Myelopoiesis is regulated by a variety of **cytokines called interleukins (IL) and colony stimulating factors (CSF)**:

- IL-3 affects many marrow cell types and provides proliferative and survival signals.
- GM-CSF (granulocyte/macrophage colony stimulating factor) promotes proliferation and differentiation of a number of early precursors but also impacts T cells and other cell types.
- **G-CSF** (granulocyte-CSF) promotes proliferation, faster maturation of neutrophils, and activates them for phagocytosis and killing.

G-CSF and GM-CSF are sometimes used clinically to raise the neutrophil count in neutropenic patients.

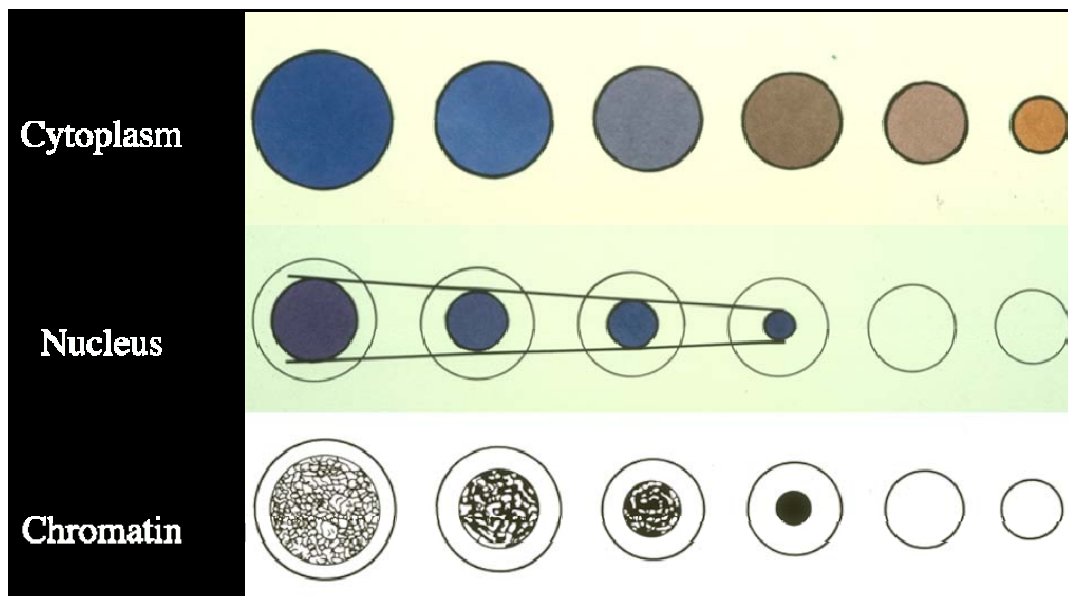


Figure 1.19. Schematic of erythroid maturation.

In the erythroid lineage, cells mature through a morphologic continuum that is difficult to consistently subdivide, so for our purposes, we will think of these cells as early, middle, and late erythroid precursors. As in Figure 1.17, many texts refer to these cells as “erythroblasts” throughout maturation, which can be confusing as they do not look like “blasts” as we usually think of them. In general, **as erythroid cells mature from the dark blue staining cytoplasm and blast-like nucleus of the proerythroblast (Figure 1.17) to the mature enucleated RBC, the cytoplasm is gaining hemoglobin (appearing more pink in Wright stains) while the nucleus is shrinking and chromatin becoming more coarse and clumpy (Figure 1.19).** Note how the erythroid nucleus is distinctive in that it is almost always perfectly round and has a very blue and white, patchy, or “salami-like” chromatin pattern, in contrast to lymphocytes and other cells, which tend to lack these clear dark and white staining areas. On biopsy sections, erythroid nuclei tend to look like round, very dark marbles. As mentioned above, immediately after the nucleus is “evicted” from the cell, residual RNA causes these reticulocytes to appear more purple in color than the mature RBC.

The production of RBC is regulated largely by the cytokine erythropoietin, sometimes referred to as “Epo” and commonly used in recombinant form to treat some types of anemia. EPO is produced by specialized cells in the kidney in response to decreased oxygen levels (Figure 1.20). A decrease in RBC numbers, for example after acute blood loss, will result in decreased O₂ levels in the kidney. Other conditions such as a hemoglobin that binds tightly to O₂ (e.g., fetal Hgb) or chronic hypoxemia could give the same signal. Serum erythropoietin levels can be measured, and can provide a clue as to the etiology of a patient’s anemia (or cause for an increase in RBC) (see Chapter 2).

C. Stem Cells, Bone Marrow Failure, and the Effects of Cytopenias

Let us pause momentarily to discuss the LT-HSC and the implications and possibilities that “stem” from its existence. **One property that defines the LT-HSC is its ability to renew itself for the life of the host animal.**

In other words, when the LT-HSC divides, one daughter can go on to produce a large cohort of differentiated hematopoietic cells but the other must remain as an LT-HSC. Since these cells intrinsically possess self-renewal properties, **genetic damage that results in a proliferative or survival advantage, or an inability to differentiate properly could be replicated and appear in multiple lineages of hematopoietic cells, with the potential for creating a leukemia. On the other hand, damage to the small stem cell pool, from immunologic attack, radiation, drugs, or toxins can lead to a failure of the marrow to create blood cells.** In either case, the

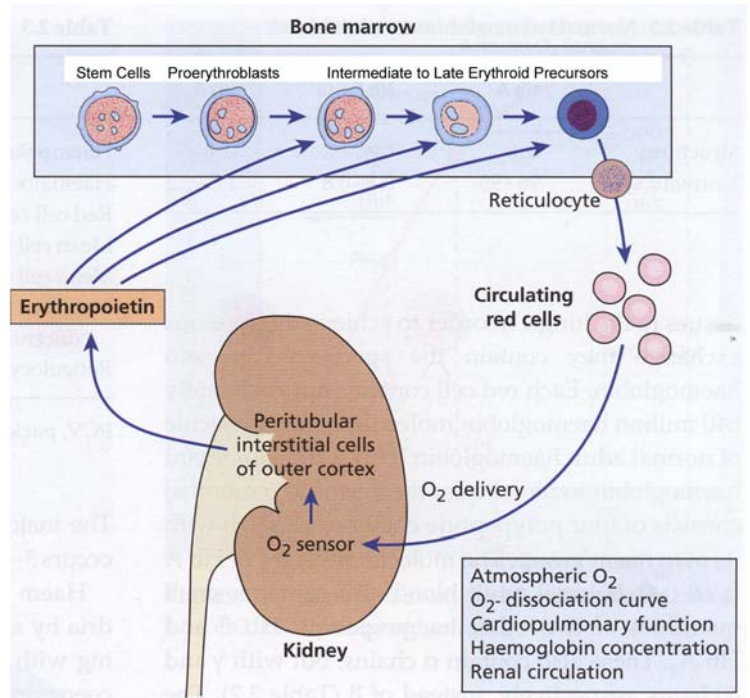


Figure 1.20. Feedback loop control of erythropoiesis mediated by erythropoietin production in the kidney in response to decreased in oxygen concentration. Adapted from Hoffbrand et al. *Essential Haematology*, 5th Ed.

damaged stem cells might require replacement, for example by allogeneic stem cell transplant (discussed below).

FYI

It is thought that LT-HSC reside in specialized “niches” in the bone marrow, likely adjacent to osteoblasts or endothelial cells, where they receive signals that promote survival, self-renewal, and when appropriate, expansion or migration into the peripheral blood stream. Most LT-HSC are quiescent in the marrow, becoming active in hematopoiesis in “waves”. Small numbers of LT-HSC also are present in the peripheral blood at all times (their numbers can be increased by use of cytotoxic drugs and cytokines). There are a couple of practical implications arising from the biologic behavior of LT-HSC. First, it is possible to harvest HSC from the peripheral blood for use in bone marrow transplantation. Second, damage to dividing cells, such as occurs with most chemotherapeutic regimens or radiation exposure, does not kill all LT-HSC. If the marrow damage is extensive enough, however, the remaining HSC will not be able to produce enough mature hematopoietic cells in time to prevent bleeding (lack of platelets) or infection (lack of WBC) and thus the patient needs to be “rescued” with additional bone marrow or HSC cells.

Table 1.3 lists the some of the signs, symptoms, and etiologies for increases or decreases in numbers of peripheral blood cells. There are two important points to take away on this issue:

1. What does the CBC tell us about the possible cause of a cytopenia?
2. What does the lifespan of the different cell types tell us about the time frame of the disorder?

Recall that the peripheral lifespan of RBC is 120 days, platelet lifespan is about 1 week, and PMN lifespan about 5 days. It takes about 2 weeks to go from HSC to mature cell in the marrow. Thus, if your HSC stopped producing offspring two weeks ago, and today’s marrow output was its final effort, it would take a few days for your PMN count to drop to near zero, about 1 week for your platelets to do the same, but over 100 days for your RBC to disappear (your RBC count and Hgb would drop about 1% per day). If a patient presents with an isolated cytopenia, this suggests that the etiology is not due to complete marrow failure. On the other hand, if all three lineages (PMN, platelet, and RBC) are decreased, marrow failure is more likely, whether it is due to replacement of marrow by neoplastic cells, a nutritional deficiency that affects all lineages (e.g. vitamin B₁₂), toxin, or stem cell failure.

As we noted above, **the reticulocyte count can be helpful in distinguishing these possibilities as well. Normally it is about 50,000 / μ l (1% of 5 million RBC). If it is increased in an anemic patient, it suggests a normal response to bleeding, hemolysis, or recovery from marrow suppression. If the reticulocyte count is not increased in such a patient, that suggests that either the marrow is not capable of responding to decreases in RBC (and possibly other cell types) or that there has been a failure of compensatory erythropoietin production.**

Potential causes of—and clinical problems associated with—increases and decreases in numbers of various blood cells are summarized in Table 1.3

Aplastic anemia

Aplastic anemia is a disease in which the stem cell compartment fails to maintain bone marrow production. In this disorder, we expect to see declines in all cell counts (**pancytopenia**) with low reticulocyte counts. A bone marrow biopsy shows near complete replacement of hematopoietic cells by adipocytes (fat) (Figure 1.21). Aplastic anemia may be caused by hereditary disorders that usually present in childhood or in young adults (e.g., Fanconi anemia), or may present as an idiopathic disorder later in life. Many of these “idiopathic” cases may be due to autoimmune attack on the stem cell population. Secondary aplastic anemia can be caused by toxic damage to the marrow by radiation or chemicals (benzene, DDT, chemotherapy drugs, gold, etc.) or secondary to viral infection (e.g., EBV).

Treatment of aplastic anemia consists of removing the offending agent, if known, and supportive therapy such as antibiotics and transfusions of RBC and/or platelets. Some patients respond well to immunosuppressive therapy such as cyclosporine, high dose glucocorticoids, or antithymocyte globulin. Definitive treatment for intractable aplastic anemia may include allogeneic bone marrow transplantation (BMT). In this procedure, the patient is prepared by immunosuppressive and bone marrow suppressive chemotherapy and/or radiation followed by infusion of HSC, preferably from an HLA-identical sibling. This can result in 80% long term survival. In the absence of an HLA-matched sibling, allogeneic BMT can also be performed using an HLA-matched, unrelated donor or stem cells derived from umbilical cord blood.

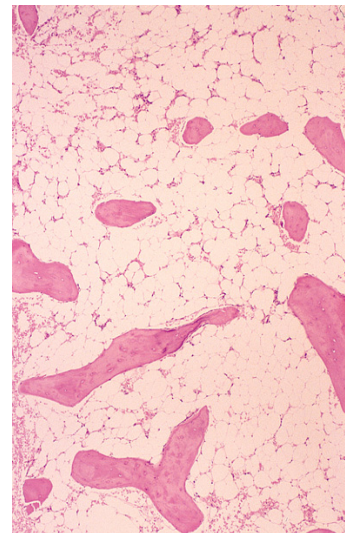


Figure 1.21. Marrow biopsy in aplastic anemia.

FYI

The use of high-resolution DNA-based HLA typing has decreased the risk of engraftment failure and immune attack of the recipient by the donor’s cells (graft-versus-host disease, GVHD) formerly associated with stem cell transplants from unrelated donors.

| Table 1.3 Increases and Decreases in Peripheral Blood Cells | | | |
|--|-------------------------------|--|--|
| Cell Type | Increase/Decrease | Signs/Symptoms | Possible Etiologies |
| RBC | ↓ Anemia | Fatigue, pallor, dyspnea | Nutritional (Fe, B₁₂, folate, Cu) Toxin (Pb) Infection Destruction (hemolysis) Bleeding Marrow failure (leukemia, storage disorder, metastases, aplastic anemia) |
| RBC | ↑Erythrocytosis | Plethoric appearance, hyperviscosity with hypoxia and/or clotting | Dehydration Hypoxia Myeloproliferative disorder (polycythemia vera) Ectopic EPO production |
| Platelet | ↓ Thrombocytopenia | Petechiae Bleeding | Destruction by consumption (e.g., hypersplenism) or autoimmune process Marrow Failure |
| Platelet | ↑Thrombocytosis | Bleeding (due to defective platelet function) or clotting | Infection Fe deficiency Myeloproliferative disorder Paraneoplastic |
| PMN | ↓Neutropenia | Susceptibility to infection (bacteria and fungi) | Overwhelming infection Autoimmune Marrow failure |
| PMN | ↑Neutrophilia | Usually none unless very high | Infection Inflammation Metabolic Cancer Drugs (steroids) Myeloproliferative disorder |
| Lymphocyte | ↓Lymphopenia | Susceptibility to infection (viral and other) | Inherited immunodeficiency Drug (steroid) Infection (e.g., HIV) Marrow failure (long term) Paraneoplastic Autoimmune disease |
| Lymphocyte | ↑Lymphocytosis | None | Neoplastic Infection (EBV, Bordetella, others) Drugs Smoking Thyrotoxicosis |

IV. Hematopoietic growth factors in clinical use

A. Erythropoietin

Erythropoietin is a glycoprotein produced by the peritubular interstitial cells of the kidney under control of a single gene on chromosome 7. It is a heavily glycosylated protein of 166 amino acids with a molecular weight of 34-39 kDa. It activates specific receptors on bone marrow cells to stimulate the division and differentiation of committed erythroid progenitor cells, thereby causing increased red cell production. Recombinant erythropoietin is produced in mammalian cell culture systems by Chinese hamster ovary cells. There are small differences in the glycosylation pattern of the recombinant erythropoietin compared to natural human erythropoietin but these appear not to have any effect on pharmacokinetics or potency.

Therapeutic uses:

- Patients with chronic renal failure do not produce enough erythropoietin and are likely to respond to treatment with exogenous erythropoietin.
- Routinely used for patients with anemia due to renal failure. Also used to treat selected patients with bone marrow depression due to chemotherapy, AIDS, bone marrow transplantation, cancer, and chronic inflammation.
- **Administration of recombinant erythropoietin should be guided by the patient's hemoglobin level. The target hemoglobin should be between 10-12 g/dL for most clinical indications. Targeting higher hemoglobin levels has been associated with an increased incidence of cardiovascular complications and increased mortality.**

B. Granulocyte colony-stimulating factor (Filgrastim)

Granulocyte colony-stimulating factor (G-CSF) is a 175 amino acid glycoprotein that stimulates the production and proliferation of myeloid progenitor cells by interacting with specific cell-surface receptors. G-CSF increases total neutrophil counts, including mature, banded, and precursor neutrophils, without increasing the number of basophils, eosinophils, or monocytes. The therapeutic form is a non-glycosylated recombinant human protein produced in *Escherichia coli* bacteria into which has been inserted the human gene for G-CSF. Also available conjugated to monomethoxypolyethylene glycol (PEG) as pegfilgrastim. This form has a half-life 4-20 fold longer than filgrastim itself.

Therapeutic uses:

The introduction of G-CSF in 1991 was a milestone in the treatment of chemotherapy-induced neutropenia. The drug reduces the duration of neutropenia and the incidence of infection in patients receiving myelosuppressive chemotherapy or bone marrow transplantation. The drug is also used to mobilize hematopoietic stem cells for collection, for treatment of patients with severe chronic neutropenia, and to reverse treatment-associated neutropenia in AIDS patients.

CHAPTER 2

OVERVIEW OF ANEMIA AND IRON

Key Concepts:

- Kinetics of red cell production and turnover
- Definition and clinical manifestations of anemia
- Iron metabolism and its relationship to iron deficiency and overload.
- Pathophysiology of anemia associated with inflammation and renal failure.

Learning Objectives:

1. Describe the normal lifespan of a red cell.
2. Define anemia and describe its clinical consequences, and describe the ways in which the body compensates for anemia.
3. Be able to use the reticulocyte count, marrow cellularity, and G:E ratio to assess red cell production and help determine the cause of anemia.
4. Describe normal iron metabolism and list the factors that affect iron balance.
5. Diagnose iron deficiency and distinguish it from other causes of microcytic anemia.
6. List the most important causes of iron deficiency.
7. Describe the pathophysiology and clinical consequences of iron overload.
8. Describe the pathophysiology of anemia of inflammation and the anemia associated with renal failure.

Part 1: THE ANALYSIS OF ANEMIA

I. Introduction

Anemia is a common medical problem, and its pathophysiology is well understood. This chapter gives an overview of methods of classifying anemia. Later chapters will take up specific types of anemia in more detail.

II. Red Cell Differentiation and Maturation

This subject is covered in more detail in chapter 1. Approximately eight days elapse from the proerythroblast stage to the release of a mature red cell into the circulation—five days in division and three days in reticulocyte maturation. During its growth period, the red cell precursor divides three to four times at about 24-hour intervals. It synthesizes hemoglobin and changes color on staining from deep blue (RNA) to pink (hemoglobin) and decreases in size. The nucleus then undergoes involution and is extruded, engulfed and digested by an adjacent macrophage.

When the late erythroblast extrudes its nucleus, it is called a **reticulocyte**. Reticulum is a collection of RNA-rich mitochondria and ribosomes that appears as nodular blue strands when stained by supravital dyes such as new methylene blue. Reticulocytes require about four days to mature into mature red cells—about three days of this period is normally spent in the marrow and one day in the peripheral blood. Approximately 1% of the circulating red cells are replaced daily (the red cell life span is about 120 days), and the newly released erythrocyte is identifiable as a reticulocyte for about 24 hours. As a result, about one percent of circulating red cells are normally reticulocytes. **The normal absolute number of reticulocytes in a person with a normal red count of 5,000,000/ μ l is therefore around 50,000/ μ l.**

Almost 25% of hemoglobin is synthesized during the reticulocyte stage. Hemoglobin synthesis stops when the hemoglobin has reached the astonishing concentration of 340 grams per liter of red cells. During this time, the RNA (reticulum) is degraded. Morphologically, the reticulocyte changes from a large irregular spherical cell with redundant membrane to the familiar biconcave disc form.

Young reticulocytes in the marrow display abundant RNA. They have bluish cytoplasm (polychromatophilia) when stained with ordinary Wright or Giemsa stains. The more mature reticulocytes normally present in peripheral blood are indistinguishable from mature red cells with Wright or Giemsa stains. Hence, **the appearance of larger bluish red cells in the peripheral blood signals early release from the marrow and suggests increased erythropoietin activity**. When seen in the peripheral blood smear they are called "polychromatophilic cells" or "shift cells."

During normal erythropoiesis, approximately 10% of the red cell precursors are destroyed in the marrow. Exaggerated intra-marrow cell death (**ineffective erythropoiesis**) occurs in certain marrow disorders such as vitamin B12 or folic acid deficiency (discussed in Chapter 3), thalassemias (Chapter 5), and myelodysplastic syndrome (Chapter 8).

A. Erythropoietin

Red cell production is regulated by erythropoietin. The blood level of erythropoietin varies inversely with the hematocrit (Fig. 2.1). When the hematocrit falls below 20%, the level normally increases by a factor of 100 to 1,000. In patients with autonomous (erythropoietin-independent) production of red cells (polycythemia vera – see chapter 8), erythropoietin falls to low levels. Erythropoietin is produced by kidney peritubular cells that sense tissue oxygen levels. Low pO_2 in the renal microvasculature occurs with anemia, acute blood loss, and conditions that shift the oxygen dissociation curve to the left (decreased oxygen release). Kidney disease often causes anemia due to decreased erythropoietin production. Extrarenal sources of erythropoietin also exist. Thus, months after total nephrectomy, erythropoietin is found again in serum, and the hematocrit gradually rises to the high 20s. Extrarenal erythropoietin probably comes from the liver, which is also a source of this hormone in the fetus.

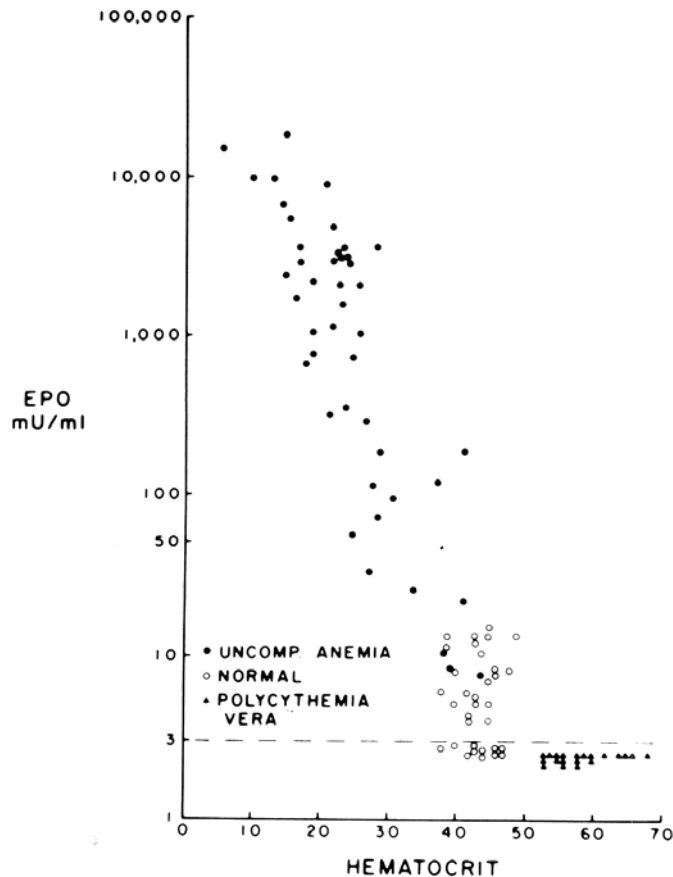


Figure 2.1. The relationship between hematocrit and plasma erythropoietin titers. Patients with anemia not complicated by kidney disease or inflammation, normal subjects and patients with polycythemia vera are indicated (from Erslev, AJ, et al.: Plasma erythropoietin in health and disease, *Ann Clin Lab Sci.* 10:250).

Erythropoietin acts on the marrow in several ways. Its predominant effect is to increase differentiation of proerythroblasts from stem cells, leading to increased output of red cells. Erythropoietin also accelerates the maturation of erythroblasts, shortening the intermitotic interval and increasing the rate of hemoglobin synthesis. Finally, increased erythropoietin promotes the release of reticulocytes at an earlier stage of development. Hence, the presence of large young reticulocytes (polychromatophilic macrocytes or shift cells) in peripheral blood is a useful sign of increased erythropoietin.

B. Lifespan and Death

Normal human red cells circulate for about 120 days, after which they are destroyed by macrophages in the spleen or, if the spleen is absent, in the liver. Hemoglobin is then recycled. Hemoglobin catabolism will be discussed at length in Chapter 4.

C. Erythrokinetics.

Normally, production of new red cells and removal of old cells are exactly balanced so that the hematocrit is stable. **Decreases in hematocrit or hemoglobin concentration, if changes in plasma volume are excluded, are due to decreased red cell production, increased red cell destruction, or to blood loss.**

1. The Granulocyte-to-Erythrocyte Ratio

The G:E ratio is the ratio of granulocytic precursors to nucleated red blood cells in an aspirate of bone marrow. Since it is a ratio, it is useful for assessing erythroid activity only when marrow granulocyte production is normal. In the normal state, the G:E ratio is about 3:1. The ratio provides an estimate of erythroid marrow activity. For example, when an increase in erythroid activity is sufficient to double or triple output of erythrocytes from the marrow, this ratio decreases to about 3:2 or 1:1, respectively. Conversely, when erythrocyte output falls by 50%, the ratio changes to 6:1.

2. The Reticulocyte Count

The enumeration of reticulocytes is the most useful everyday measure of effective marrow production (i.e., the release of newly made red cells from the marrow). The release of one reticulocyte signals effective production of one red cell by the marrow. In contrast, the G:E ratio represents total red cell production, including defective red cells that are not destined to be released into the blood. **If the number of red cell precursors in the marrow is increased but the reticulocyte count is not, ineffective erythropoiesis is present.** The clinical laboratory reports reticulocytes in two ways: as a percentage of circulating red cells, and as the absolute number of reticulocytes/ μl of blood (red count \times % retics). The absolute reticulocyte count is normally about 50,000/ μl ($1.0\% \times 5 \times 10^6$ erythrocytes/ μl). A correction is then made to account for changes in the maturation time of reticulocytes in peripheral blood. **When erythropoietin production increases in response to anemia or hypoxia, reticulocytes are released from the marrow prematurely (Fig 2.2). This increases the time a reticulocyte spends in the blood, and inflates the absolute number of reticulocytes.**

In a patient whose hematocrit is less than about 30%, reticulocytes are released from the marrow about 24 hours earlier than normal. It then takes about 2 days instead of the usual one day for the reticulocyte to lose its reticulum and become a mature red cell, thereby doubling the reticulocyte count. Since a normal bone marrow should be able to increase red cell production *at least* two-fold in the face of anemia, this means that the absolute reticulocyte count (including both mature and immature reticulocytes) should go up about 4-fold (i.e., to at least 200,000) under these circumstances. This assumes the marrow has had time to manufacture new red cells, which takes about a week. **In an anemic patient, an absolute reticulocyte count of greater than 200,000 is a good indication that the anemia is not due to inadequate red cell production. Very high reticulocyte counts (over 300,000) usually indicate the presence of chronic hemolysis (see figure 2.3 and chapter 4). On the other hand, a low reticulocyte count (below 100,000) in an anemic patient is usually an indication of inadequate red cell production.**

Some patients with mild hemolysis (shortened RBC survival) or chronic minor blood loss are able to replace lost RBC fast enough to keep from becoming anemic. In the case of hemolysis, this is called “compensated hemolysis.” These individuals will have an elevated reticulocyte count. **In a patient with a normal (or nearly normal) hematocrit, an absolute reticulocyte count of over 100,000 usually indicates the presence of hemolysis or (less likely) ongoing blood loss.**

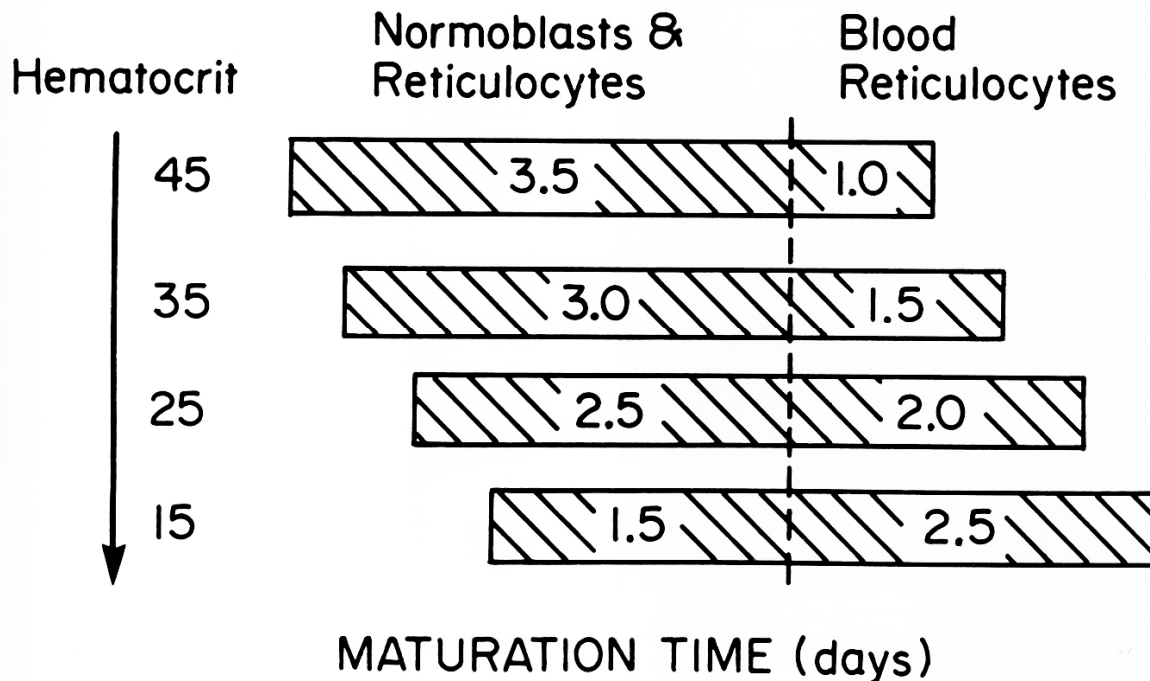


Figure 2.2. The time red cell precursors (“normoblasts”) and reticulocytes spend in the marrow and blood at different hematocrits. The horizontal dashed line indicates the marrow barrier. As the hematocrit decreases, there is progressively shorter marrow time and longer blood maturation time. (From Bothwell TH, et al: *Iron Metabolism in Man*. 2nd Edit, p. 238 Oxford, Blackwell).

III. Pathophysiology of the Anemias: An Overview

We can measure an individual’s complement of red cells in 3 ways:

1. The **red blood count**: Red cells are counted electronically as they pass through a calibrated orifice and reported as the number of red cells/ μ l of whole blood.
2. The **hemoglobin**: Hemoglobin is determined by lysing the red cells in a given volume of whole blood and measuring the free hemoglobin spectrophotometrically. It is expressed as grams/dl.
3. The **hematocrit**: The hematocrit can be measured by centrifuging a sample of whole blood in a narrow tube and determining the volume of the sample occupied by the red cells. It is expressed as a percentage. This method is not used in the clinical laboratory, where the hematocrit is calculated by the analyzer from the red count and the MCV.

Because the hemoglobin is directly measured by the analyzer and is a more accurate determination than the red blood count, “hemoglobin” is the best way to express the quantity of oxygen carrying capacity. In this book (and everywhere) hemoglobin and/or hematocrit are commonly used interchangeably.

The concentration of hemoglobin, the volume of red blood cells per unit volume of blood (hematocrit), and the number of red cells per unit volume of blood (RBC count) are closely regulated. Actual values, which differ with age and sex, are given in Chapter 1, Table 1.1. A decrease below these levels is termed "anemia," while an increase is called "erythrocytosis."

Anemia is defined as a red cell mass that is below the normal range for individuals of the same age and (in the case of adults) sex. Since measurements of red cell mass are not generally available, **for practical purposes a hemoglobin, hematocrit or RBC count below the age- and sex-specific normal range generally indicates the presence of anemia.** It is important to remember, however, that changes in plasma volume (hemodilution or hemoconcentration) can lower or raise the hemoglobin concentration without affecting total red cell mass. On the other hand, recent acute blood loss can lower red cell mass dramatically without changing the hemoglobin level because red cells and plasma are lost in equal amounts. It may take several hours before enough interstitial fluid enters the vascular space to dilute the remaining red cells and lower the hemoglobin.

A slow decline in the hematocrit to 30% usually produces no symptoms in normal sedentary subjects. However, the capacity for aerobic activity such as competitive athletics or heavy work is sharply limited by even mild anemia. When the hematocrit falls below 30%, weakness, fatigue, and breathlessness are common. Hematocrits below 25% are poorly tolerated, especially in the elderly, and transfusions may be required. Anemia may critically limit oxygen delivery to organs whose blood supply has been compromised by vascular disease. For example, patients may have chest pain (angina) or pain in the legs (claudication) when the hematocrit falls below 30%, but not when the hematocrit is higher.

Acute blood loss does not immediately change the measurements of red cells, as red cells and plasma are lost in equal amounts. Symptoms and signs are related to the volume of blood lost. Tachycardia and drop in blood pressure occur when the amount is more than 20% of the blood volume (1 liter in a 70 kg adult). If more than 30% of the blood volume is acutely lost, air hunger, cold clammy skin, thready pulse, and other signs of shock appear. If more than 50% of the blood volume is lost, death will occur unless the blood volume is replaced immediately.

Compensatory mechanisms for anemia include an increase in heart rate and left ventricular stroke volume. Also, under the influence of increased levels of 2,3 diphosphoglycerate (2,3 DPG – see chapter 4), the remaining red cells shift the oxygen dissociation curve to the right and unload more oxygen to the tissues.

A. Normal Response of Marrow to Anemia

The normal marrow acts to reverse anemia. Acute anemia causes a drop in oxygen transport to the kidney and a logarithmic rise in erythropoietin. This, in turn, leads to the appearance of extra reticulocytes in the blood within 12 hours (due to release of immature reticulocytes, as discussed above). While this influx of new cells does not raise the hematocrit significantly, it does show that the erythropoietin level is responding. The rise in erythropoietin also causes an increase in the rate of differentiation of erythroid cells from their precursors.

One or two days after the abrupt onset of anemia from bleeding or hemolysis, the number of early erythroid forms increases in the marrow, followed over the next couple of days by cells in later stages of development. By five to seven days, the reticulocyte count increases significantly and, after seven to ten days, the marrow response is complete and a new plateau of production is established. If the erythropoietic stress is severe and ongoing, erythroid marrow expansion may go on for months. The degree of response is proportional to the severity of the anemia. This is shown in Fig. 2.3, in which output of red cells by the marrow is expressed in multiples of basal red cell production. These normal responses of the marrow to anemia require an adequate supply of iron, vitamin B₁₂, and folic acid. If the nutritional supply is marginal, the response will be blunted.

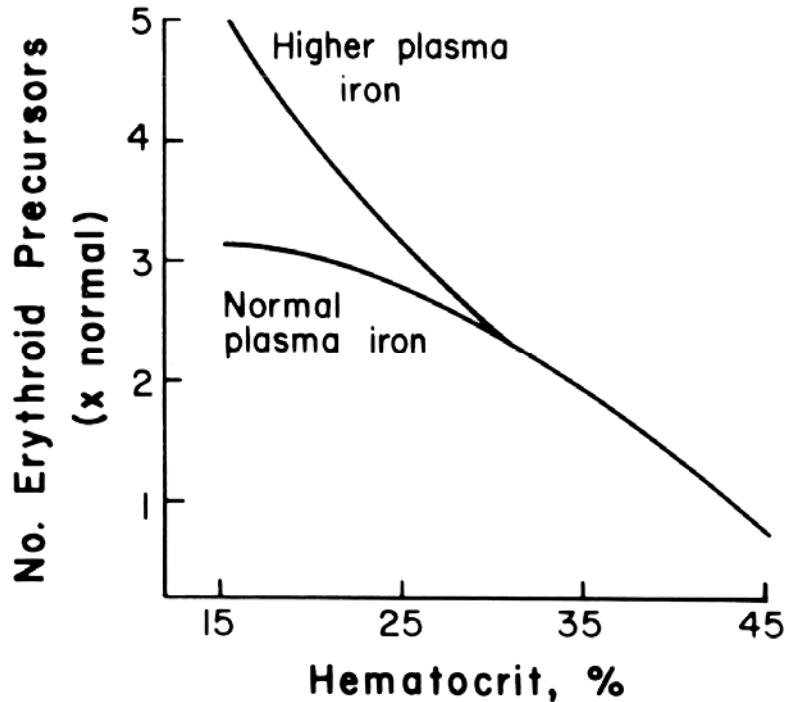


Figure 2.3. The response of normal erythroid marrow to anemia. The response depends on adequate iron availability. **The higher levels of response (five or more times normal) are not observed unless serum iron is higher than normal, as in hemolysis.**

When the marrow expands, it encroaches on marrow fat. If the expansion is marked and long-lasting, the erythroid marrow expands into bones that do not normally contain active marrow. If marked and persistent expansion occurs during childhood, bone structure is altered to accommodate the increased mass of marrow. This is discussed further in Chapter 5.

B. Analysis of Anemia

The two most useful factors to consider in the initial evaluation of an anemic patient are the reticulocyte count (an indication of marrow response) and the appearance of the red cells (size and shape), which may give important clues as to the cause of the anemia. The reticulocyte count is interpreted according to the guidelines listed above. Interpretation of red cell size and shape is discussed below.

1. Red cell size

Red cells can be characterized by their volume.

- **The mean corpuscular volume (MCV) is the hematocrit (volume) divided by the red cell count. This value is very useful in the primary classification of anemia.** It is typically expressed in units of 10^{-15} liters, or femtoliters (fl).

Two other indices, the mean corpuscular hemoglobin ($MCH = \frac{Hgb}{RBC}$) and the mean

corpuscular hemoglobin concentration ($MCHC = \frac{Hgb}{Hct}$) are less useful diagnostically and

will not be discussed further. The example below shows the calculation of MCV for a patient with a hematocrit of 40 and a red cell count of $4 \times 10^6/\mu\text{l}$:

Hematocrit = 40 = 0.4 liters/liters blood

Red blood count = 4×10^6 rbc/ 10^{-6} liters blood = 4×10^{12} rbc/liter blood

$MCV = \frac{0.4 \text{ liters/liters blood}}{4 \times 10^{12} \text{ rbc/liter blood}} = 1 \times 10^{-13} \text{ liters/rbc} = 100 \text{ fl/rbc}$

OR divide Hct by RBC and multiply by 10; $40/4 \times 10 = 100$

When red cells are small (MCV<80), poor hemoglobin production is the basic problem. When red cells are large (macrocytic; MCV>100 fl), defects in DNA synthesis or brisk reticulocytosis are most likely. Subsequent chapters will discuss conditions that can cause microcytic and macrocytic anemias.

2. Red cell shape

A second part of the analysis of anemia is to study the blood smear for red cell shape changes. Many different abnormal shapes (sickle cells, spherocytes, broken cells, targets, etc.) may be seen. **Anisocytosis is the term for variation of red cell size. When red cells of many different shapes are seen, the term poikilocytosis is used.** In addition, there may be abnormalities of platelets or white cells that give important clues to the cause of anemia. Abnormalities of red cell shape associated with specific types of anemia will be discussed in subsequent chapters.

C. The Kinetic Classification of Anemia

Anemias may also be classified by the gross rate of production of red cells (G:E ratio) and the by effective release of red cells to the circulation (absolute reticulocyte count). This classification

has four parts: decreased production, decreased delivery, increased destruction, and blood loss.

1. Decreased production (hypoproliferative anemia)

In hypoproliferative anemia, the rate of production of erythrocytes by the marrow is lower than is expected for the degree of anemia. In industrial terms, the car dealers are calling for fifty thousand pickup trucks but the assembly line is only delivering ten thousand. The reticulocyte count is less than 200,000/ μ l (usually substantially lower—under 100,000/ μ l) and the marrow erythroid cellularity is normal or reduced, in proportion to the reticulocyte count. Aplastic anemia (Chapter 1) is a particularly serious form of hypoproliferative anemia in which red cell production almost totally fails. Other kinds of hypoproliferative anemia to be discussed in this and subsequent chapters include iron deficiency, anemia associated with chronic inflammation or renal disease, and anemias due to marrow infiltration or replacement by malignant cells.

2. Decreased delivery (ineffective erythropoiesis)

In ineffective erythropoiesis, developing erythrocytes are destroyed (typically via apoptosis) within the marrow. In industrial terms, the factory is running a full assembly line, but few trucks are coming off the ramp because they are rejected by the inspectors. The reticulocyte count is less than 200,000, but the marrow erythroid cellularity is disproportionately high. Examples of anemia associated with ineffective erythropoiesis include folic acid and B₁₂ deficiency (Chapter 3), the thalassemias (Chapter 5), and myelodysplastic syndromes (Chapter 8).

3. Increased destruction (hemolytic anemia)

Hemolytic anemia means that, after leaving the marrow, the red cells have a markedly shortened life span—perhaps 10-30 days in circulation instead of the normal life span of 120 days. In industrial terms, the assembly line is going night and day because the trucks last only a year instead of ten. The reticulocyte count is high (often greater than 300,000/ μ l) and there is a proportionate increase in marrow erythroid cellularity. Anemia occurs when the rate of hemolysis exceeds the marrow production capacity. Lesser degrees of hemolysis are characterized by reticulocytosis (reticulocytes > 100,000/ μ l) in the absence of anemia, called **compensated hemolysis**. Examples of hemolytic anemia to be discussed in this course include hereditary spherocytosis (Chapter 4), sickle cell disease (Chapter 5), autoimmune hemolytic anemia (Chapter 7), and glucose 6-phosphate dehydrogenase deficiency (Chapter 4).

4. Blood loss anemia

Blood loss anemia is due to external or internal bleeding. When blood loss is acute, the blood counts do not accurately reflect the loss of red cells for 24 to 72 hours, which is the time required to re-expand the blood volume by mobilizing extracellular water and plasma proteins (Fig. 2.4). Seven to ten days are required for the bone marrow to reach the level of production demanded by any acute anemia. The reticulocyte count is increased, although not generally to the degree seen in hemolysis, and the marrow erythroid cellularity is proportionately increased. The marrow response to blood loss is less vigorous than the response to hemolysis because plasma iron levels are lower (Fig 2.3).

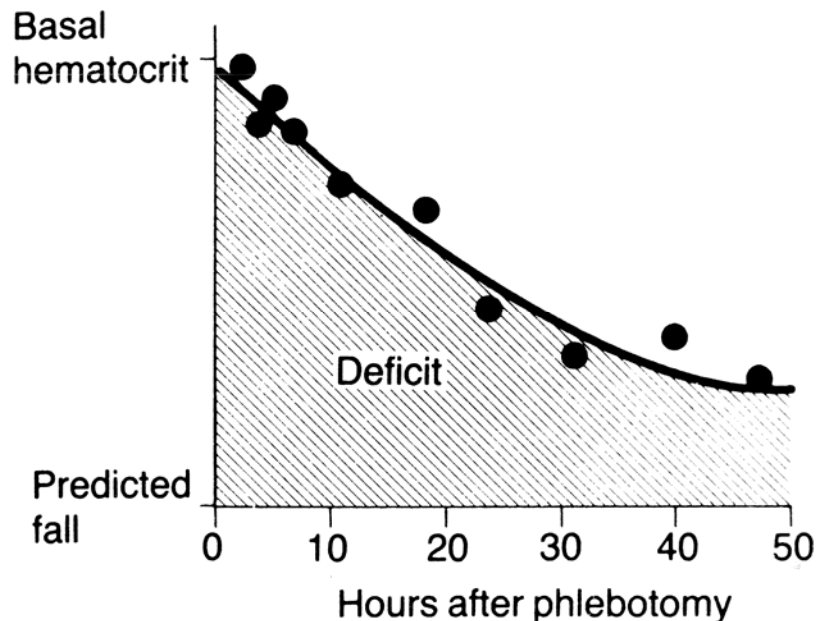


Figure 2.4. After a sudden loss of whole blood, the fall in hematocrit is a gradual process that depends on the rate of mobilization of albumin and water from extravascular sites. Full expansion of the blood volume and the lowest hematocrit value may not be reached for 48-72 hrs. (From Williams WJ, et al., *Hematology*, McGraw-Hill, 3rd Edit. pg. 668).

In practice, we take three steps in the investigation of anemia after the history and physical are completed.

1. The red cells are sized by an electronic particle counter and the MCV (mean corpuscular volume) is determined. On the basis of the MCV, anemias are classified as microcytic, macrocytic, or normocytic. Microcytic anemias are associated with defects in hemoglobin synthesis, such as iron deficiency or thalassemia. Macrocytic anemias are associated with defects in cell division such as vitamin B₁₂ or folic acid deficiency.
2. The blood smear is examined for abnormalities in the red cells and other cells as well.
3. We use kinetic analysis when the other methods are not productive, estimating red cell delivery using the absolute reticulocyte count. If this information is not sufficient to determine the cause of anemia, a bone marrow biopsy may be necessary.

IV. Summary

The biochemistry of the red cell is understood in detail. As the red cell matures, one can visualize the formation of hemoglobin and the disappearance of RNA. The nucleus is extruded and the cell is delivered the peripheral blood to circulate for four months. Thus a relatively small volume of red cell precursors, perhaps 300 cc, expands into 2,200 cc of circulating cells—about two trillion cells. Anemia represents a failure of this elegant system. It may be caused by decreased production, decreased delivery, increased destruction, or blood loss. Anemia may be analyzed by examining kinetic parameters, and by the size and shape of the erythrocytes in the blood.

Part 2: IRON METABOLISM AND HYPOPROLIFERATIVE ANEMIAS

I. Iron Metabolism

Iron is the essential element in hemoglobin and is also found in muscle myoglobin and in a variety of critical enzymes. **Iron deficiency is one of the most common problems in medicine, with 25% affected in many world populations. In adults in the United States, blood loss, not poor nutrition, is the major cause.**

A. Food Iron Availability

The average American diet contains 7 mg of iron per 1,000 calories. The person who consumes 2,000 calories will ingest about 14 mg of iron and absorb about 10% of this amount.

Roughly 10% of dietary iron is present as heme iron, which is derived from the hemoglobin and myoglobin ingested in meat, poultry, and fish. Although comparatively small in amount, it accounts for 25-33% of the iron normally absorbed, the fraction increasing further in iron deficiency. The heme molecule, with its iron atom, is absorbed intact. The iron is liberated in the intestinal mucosal cell. Absorption of heme iron is not affected by the many factors that affect nonheme iron absorption.

Non-heme iron in grains and vegetables is in various chemical forms, from which it is liberated during digestion. Availability of iron for absorption is affected by the mix of foods present, some of which enhance while others inhibit absorption. Enhancers of non-heme iron absorption include heme iron and ascorbic acid (Vitamin C). Naturally-occurring inhibitors include carbonates (soft drinks), tannate (tea and coffee), oxalate (spinach, rhubarb), phosphates, and egg yolk phosphoprotein. Many of these are anions that make iron insoluble. For example, when tea or coffee is drunk with a meal, absorption of nonheme iron falls by 50-75%. EDTA, a cation chelator present in soft drinks and prepared foods, is a powerful antagonist of absorption.

B. Gastrointestinal Tract

The duodenum is the major site of iron absorption. The amount of iron absorbed is determined primarily by the body's need for iron. The regulation of iron absorption is discussed in later paragraphs. **The body lacks any method for excreting iron. Thus, accumulation of excess body iron as a result of repeated transfusion or genetic disorders may cause iron overload and tissue injury** (see below).

C. Iron Distribution

Iron is distributed in normal adults as shown in Table 2.1. **The largest quantity is present in the form of hemoglobin iron. One milliliter of packed cells contains 1.2 mg iron.** The second largest fraction is in myoglobin. Iron is present in a large number of metalloenzymes, most notably the cytochromes and flavins. The amount of storage iron is about 1,000 mg in males, but is lower in females, ranging from 200-400 mg. The majority of storage iron is present as ferritin with the remainder, 20-30%, present as hemosiderin (denatured ferritin).

Table 2.1

DISTRIBUTION OF IRON IN NORMAL ADULTS

| | |
|--------------------------------|--------------|
| Functional compounds | |
| hemoglobin | 2,230 mg |
| myoglobin | 140 mg |
| tissue enzymes | 8 mg |
| Transport | |
| transferrin | 4 mg |
| Stores (ferritin, hemosiderin) | |
| males | 1000-1400 mg |
| females | 0-400 mg |

1 cc of red cells contains about 1 mg iron
1 cc of whole blood contains about 0.5 mg iron
1 unit of blood contains 200-250 mg iron

D. Iron Balance

In the normal steady state, iron absorption and loss are balanced. Iron in males and nonmenstruating females is lost only through desquamating cells of skin (including hair and nails), kidney, and intestine, and a small amount of intestinal blood loss (0.5 to 1 ml per day). Total iron losses average about one milligram per day in an adult male.

Iron balance is appreciably affected by growth, menstruation, pregnancy, and lactation.

During a baby's first year of life, the iron required by the rapidly expanding red cell mass and muscle may exhaust iron stores, while the diet may not yet provide sufficient iron (Fig. 2.5). This is more likely in premature infants, who begin extrauterine life with less iron. The probability of iron deficiency in babies also depends on child feeding practices. A milk diet provides relatively little iron (0.1 to 0.2 mg/dl); however, twice as much iron is available from human milk as from cow or goat's milk (unless fortified with iron). Young children are iron-deficient in many parts of the world. Surveys in the United States show that 9% of children aged 12-36 months are iron deficient with 3% having iron-deficiency anemia. These rates are significantly higher among the poor.

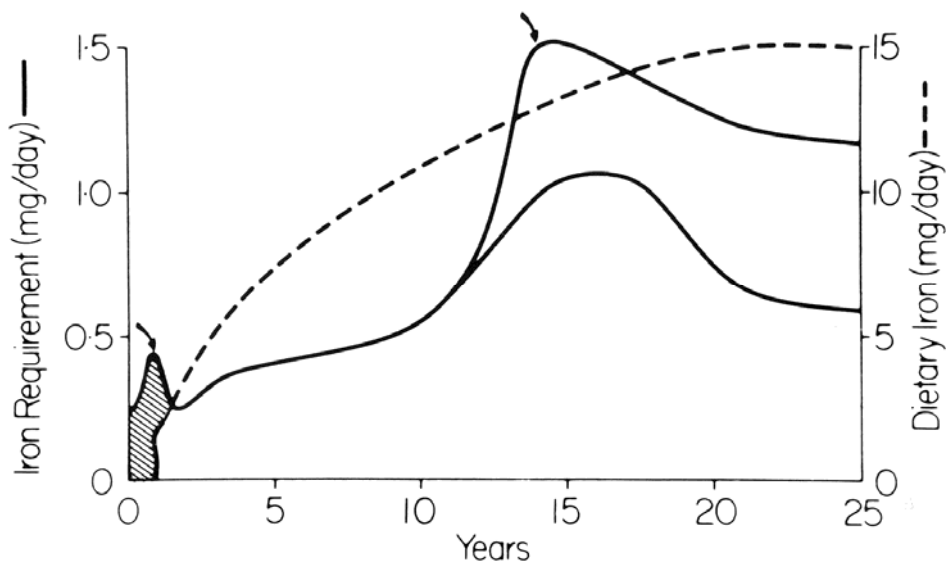


Figure 2.5. Iron requirement. The daily iron requirement at different ages is indicated by the black line. At age twelve, the line divides into the requirements for females (upper solid line) and males (lower solid line). The dashed line indicates the available iron in the normal Western diet. The shaded area during the first year of life indicates the period of negative iron balance, when the infant outgrows its iron supply. The arrow also shows the other critical period, when menstrual losses and growth needs are likely to exceed supplies. (From Bothwell, TH, et al: *Iron Metabolism in Man*, Oxford, Blackwell, 1979.)

During the reproductive years, the average menstrual blood loss is 30 ml/month. Since one milliliter of blood contains $\frac{1}{2}$ mg iron, the average monthly menstrual loss is about 15 mg iron. Thus, the average menstruating woman requires an additional 0.5 mg/day (15 mg/30 days); this requirement may reach 2.5 mg per day in those with heavy menses. Since normal absorption is 1.0-1.5 mg/d and since maximal dietary absorption is essentially capped at 3-3.5 mg/d with even an optimal diet and the stimulus of iron deficiency, it is obvious that iron balance is tenuous in women. Not surprisingly, **negative iron balance is common in women**. Surveys of menstruating U.S. women show that about 10% lack iron stores and 3-5% have suboptimal hemoglobin values due to iron deficiency. In the developing world the incidence is much greater.

The tendency toward negative iron balance in women is compounded by pregnancy and lactation (Table 2.2; Fig. 2.6). While menses cease during pregnancy, the increase in maternal red cell mass (about 400 ml red cells, 480 mg iron), the fetal and placental requirements for iron (350 to 400 mg), and the blood loss attending delivery (average of about 200 ml, 85 mg iron) amount to roughly one thousand milligrams of iron beyond basal requirements. The iron of the expanded red blood cell mass is returned to the body at the end of gestation, making the net loss about 500 mg. Therefore, **each pregnancy uses approximately 500 mg of iron beyond the normal daily requirements**.

TABLE 2.2: IRON NEEDS IN PREGNANCY

| | | |
|---|-------|--------|
| Expanded red cell mass | | 480 mg |
| Formation of fetus and placenta | | 400 mg |
| Average blood loss (without episiotomy) at delivery | | 85 mg |
| | | |
| | Total | 965 mg |
| Expanded red cell mass returned after parturition | | 480 mg |
| | | |
| | Total | 485 mg |

Lactation requires an additional 0.5 to 1.0 mg per day. **Since these demands exceed iron stores in virtually all females with even the first pregnancy, iron supplements are prescribed during pregnancy and lactation.** The prevalence of iron deficiency anemia among low-income pregnant women is 9%, 14%, and 27% during the first, second, and third trimesters, respectively. Fortunately, the low iron stores of the mother do not prevent the fetus from capturing its necessary iron except in extremely severe iron deficiency.

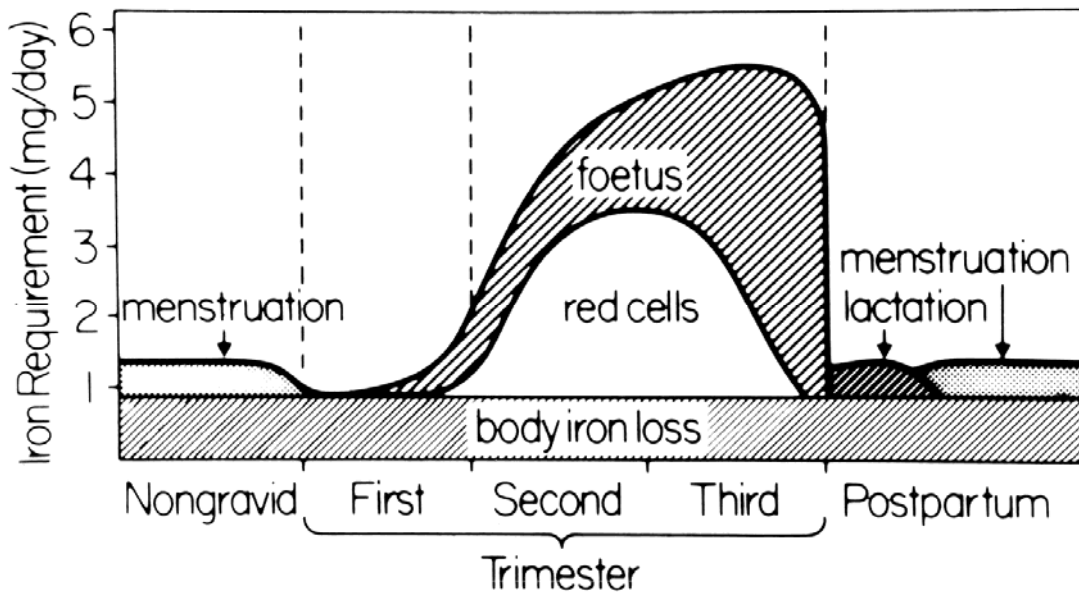


Figure 2.6. Daily iron requirements during pregnancy. The requirements for expanding the red cell mass, fetal needs, and lactation are laid on the basal daily iron loss and menstruation. (From Bothwell TH, et al.: *Iron Metabolism in Man*, Oxford, Blackwell, 1979.)

E. Ferrokinetics

Once iron enters the circulation, it remains in a closed loop, as shown in Fig. 2.7. The major features of this pathway are:

- transport through plasma
- uptake by red cell precursors in marrow
- circulation of this iron in red cells

- entrapment of senescent red cells by macrophages with salvage of iron from heme,
- return of iron from macrophages to plasma.

There is also a pool of storage iron in the macrophages that partially exchanges with the iron derived from red cell turnover. Finally, cells need a method to control uptake and disposition of iron. We will examine each of these aspects in turn.

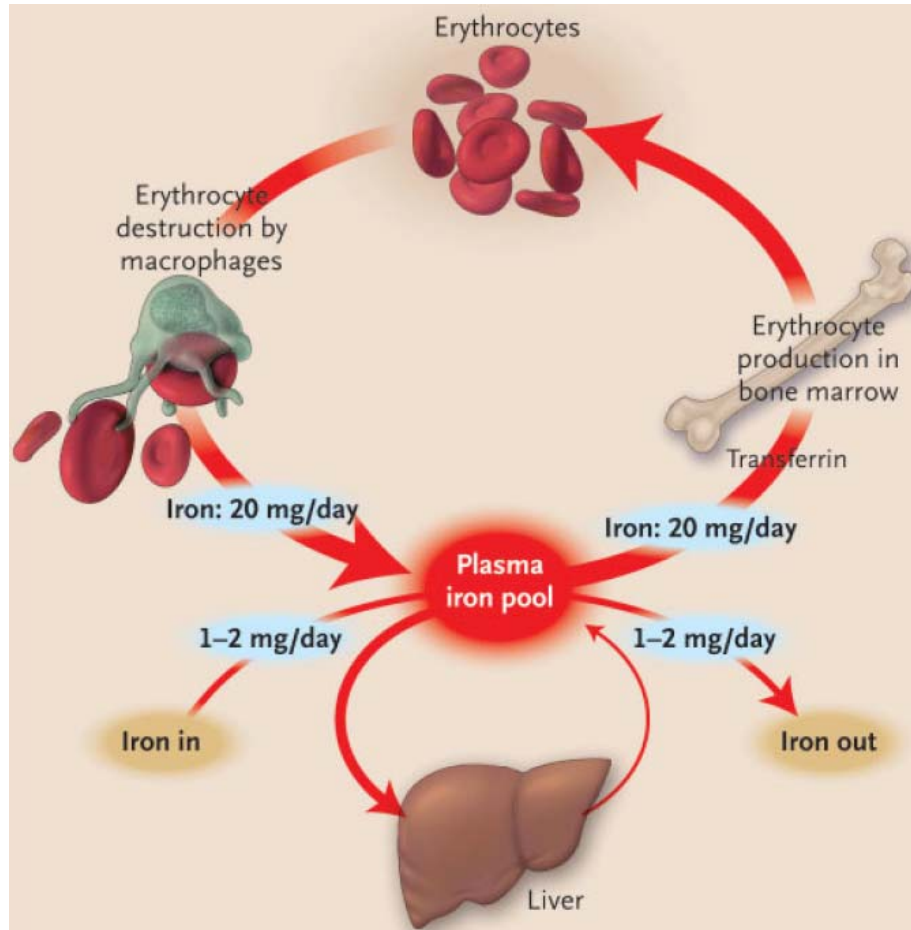


Figure 2.7. Plasma levels of iron are closely regulated to ensure a daily supply of about 20 mg to the bone marrow for incorporation into hemoglobin. Most of the iron in the plasma derives from the continuous breakdown of hemoglobin in senescent red cells by macrophages. 1 to 2 mg per day of iron is also taken up by duodenal enterocytes and transferred to the plasma compartment or, depending on body needs, stored in the enterocytes as ferritin. These stores are eliminated when enterocytes are sloughed at the end of their life cycles; apart from menstrual blood loss, this is the only significant means by which excess body iron is excreted. Iron recycled by macrophages and that absorbed from the gut is loaded onto serum transferrin and delivered primarily to the bone marrow for reincorporation into new red-cell precursors. The remaining body iron (about 1,000 mg) is stored, primarily in hepatocytes. (*N Engl J Med* 2004;350:2383)

1. Plasma transport

Ferric ions are carried in plasma by transferrin, a glycoprotein that is synthesized in hepatocytes and macrophages. Each molecule contains two iron-binding sites.

Transferrin has a very high association constant for iron ($K_a \sim 10^{24}$), which means that there is an average of about one free ferric ion in the entire circulating blood volume! This is noteworthy because ionic iron is extremely toxic. About one third of the transferrin sites are ordinarily occupied by iron. The average serum iron (SI; the clinical measure of iron-bound to transferrin in serum) concentration is about 100 $\mu\text{g}/\text{dl}$. The total iron-binding capacity (TIBC; iron bound to transferrin plus the apotransferrin iron binding capacity) is normally about 300 $\mu\text{g}/\text{dl}$.

2. Iron uptake by erythroid cells

Iron is transported by transferrin to developing erythroid precursors and reticulocytes in the bone marrow, which have a voracious iron requirement. Transferrin attaches to specific receptors, termed transferrin receptors, on the cell membrane. Over the several days of life as an erythroblast and reticulocyte, each cell takes up about one billion iron ions by this process.

3. Removal of senescent red cells and iron recycling

The fully hemoglobinized red cell then leaves the marrow and circulates for about 120 days. The next step in the iron cycle occurs at the end of red cell life. Senescent red cells are trapped in macrophages. The cell is lysed, hemoglobin is degraded, and iron is separated from the heme ring.

4. Delivery of iron to plasma

Iron is then released by the macrophage to serum transferrin and is returned to the marrow for reuse in hemoglobin synthesis. Thus **iron is recycled and avidly conserved**. Normally only 1mg of the 3,500 mg of total body iron is lost and replaced each day.

F. Iron Storage

Iron is stored in two forms, ferritin (80%) and hemosiderin (20%). Ferritin is a polyhedral protein shell containing up to 4,500 ferric salt molecules.

Ferritin conserves iron and protects cells from the highly reactive ferric ion. Ferritin also circulates in plasma in nanogram quantities. Plasma ferritin is mainly derived from macrophages, and **the plasma ferritin level normally serves as a fairly good index of body iron stores**. Ferritin is elevated in relation to stores, however, in inflammation and liver disease.

Hemosiderin is the form of storage iron visible by light microscopy. Found in macrophages in the bone marrow, liver, and spleen, it can be seen in unstained tissue sections as refractile yellow particles and as deep blue particles when stained with Prussian blue. It is probably formed by aggregation of partially denatured and deproteinized ferritin. Iron bound to hemosiderin is more difficult to mobilize than iron bound to ferritin.

II. Regulation of the Iron Cycle

There is no way for the body to excrete excess iron. Therefore iron homeostasis depends on three factors:

1. Control of the amount of iron absorbed from the GI tract.
2. Regulation of the rate of iron released to the circulation from the macrophages that are recycling senescent red cells.
3. Control of the amount of iron released to the circulation from the macrophages containing the iron storage protein ferritin.

Hepcidin, a protein made in the liver, is the main regulator of iron homeostasis; it inhibits the major iron “exporting” protein, ferroportin.

Ferroportin is responsible for moving (exporting) iron from duodenal enterocytes and macrophages into the circulation. (See Figure 2.8.)

When plasma iron levels are high, hepcidin release increases and ferroportin is inhibited; thus less iron is absorbed and less is released from macrophages. Conversely when plasma iron levels are low, hepcidin levels fall, ferroportin is uninhibited, and iron absorption and macrophage iron release increase.

As will be discussed in subsequent paragraphs, **perturbations in the hepcidin/ferroportin interaction help explain the pathophysiology of the anemia of inflammation and the iron overload syndrome, Hemochromatosis.**

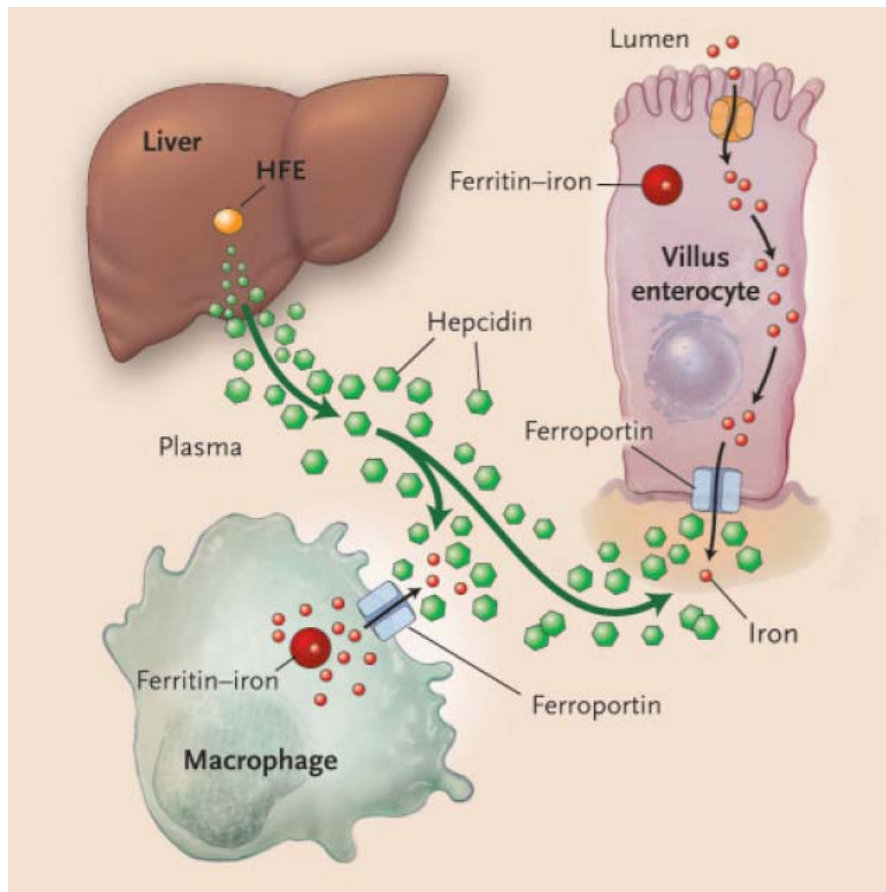


Figure 2.8 Regulation of iron uptake and storage. **Hepcidin**, a peptide produced in the liver, is a key regulator of iron release from villus enterocytes and macrophages. Hepcidin, whose production is upregulated by high plasma iron levels or inflammation, inhibits iron release from these cells and lowers GI absorption. Low iron levels decrease hepcidin production, which in turn stimulates iron absorption and release into the blood. The HFE gene modulates hepcidin production. Mutations in HFE can cause diminished hepcidin release, and can eventually cause iron overload (**hereditary hemochromatosis**). (*N Engl J Med* 2004; 350:2383.) <http://www.answers.com/topic/card-stock-2#ixzz2r30iPMHI>

III. The Hypoproliferative Anemias

The hallmark of hypoproliferation is lower than expected marrow erythroid cellularity and red cell production for the degree of anemia. Although production parameters (absolute reticulocyte count and G:E ratio) may be normal or even increased relative to levels seen in normal subjects, they are, nevertheless, *lower than expected* for the degree of anemia. In the case of anemia of sudden onset, the assessment of erythroid production must be made after anemia has been present for seven to ten days, in order to give the marrow time to respond.

The hypoproliferative anemias are due to three basic mechanisms:

- **An insufficient supply of iron for hemoglobin synthesis (iron deficiency or sequestration).**
- **Low erythropoietin levels for the degree of anemia.**
- **Marrow damage.**

A. Iron Deficiency Anemia

Iron deficiency is one of the hypoproliferative anemias: Reticulocyte production does not increase and the marrow has fewer red cells precursors than expected for the degree of anemia.

The mechanism of iron deficiency in adults is usually blood loss. Exceptions to this rule are babies whose rapid growth exceeds dietary iron availability and the patient who absorbs iron poorly because of a small bowel disorder called celiac disease or because the stomach or duodenum has been altered by surgery.

In contrast to younger women, where iron deficiency is usually a consequence of menstrual losses or pregnancy, **iron deficiency in adult men and in post-menopausal women is nearly always due to gastrointestinal blood loss.** Lesions that commonly lead to blood loss include esophagitis, ulcers of the stomach and duodenum, inflammatory bowel disease, carcinoma of the colon and stomach, and even hemorrhoids. Aspirin may also cause blood loss and iron deficiency by increasing normal gastrointestinal blood loss (0.5 ml per day) to 5 ml/day. Gastrointestinal parasites are a major cause of blood loss in many parts of the world. The serious nature of many of these disorders makes it critical to determine the cause of each patient's iron deficiency.

Iron deficiency begins with negative iron balance (Table 2.3). The typical laboratory signs of iron deficiency only appear after the stores of ferritin and hemosiderin have been completely exhausted. Transferrin receptors increase and ferritin decreases. Plasma iron falls and transferrin rises. The drop in serum iron limits hemoglobin synthesis, resulting in initially normocytic and normochromic anemia. As iron deficiency becomes more severe, red cells become smaller (reduced MCV) and the amount of hemoglobin in individual red cells falls. Such cells are recognizable in peripheral blood smears as hypochromic microcytes.

Iron deficiency affects body organ function in many ways, some overt, some subtle. Work capacity, exercise tolerance, and productivity decline in direct proportion to the decrease in hemoglobin (Fig. 2.9, Fig. 2.10). This is of considerable economic importance in developing countries, where iron deficiency is common and physical labor very important. Since iron is present in many enzymes (cytochromes, cytochrome oxidase, xanthine oxidase, catalase, succinate dehydrogenase, peroxidases, etc.), it is not surprising that iron deficiency affects tissues other than erythrocytes. Nearly half of the enzymes of the Krebs cycle contain iron or require it as a cofactor. Severe iron deficiency is associated with cheilosis (fissures at the angles of the mouth), atrophy of lingual epithelium, and brittle fingernails and toenails, which are flat or concave (spoon nails) [Fig. 2.11].

TABLE 2.3. STAGES IN DEVELOPMENT OF IRON DEFICIENCY OR INFLAMMATION

| State | Iron stores | Hgb g/dl | Fe µg/dl | TIBC µg/dl | Fe saturation, % | Ferritin µg/l | RBC morphology | EPO level | Shift cells |
|------------------------|-------------|----------|----------|------------|------------------|---------------|---|-----------|-------------|
| Normal | N | 15 | 100 | 300 | 33 | 100 | N | N | A |
| Iron depletion | D | 15 | 100 | 300 | 33 | 25 | N | N | A |
| Borderline deficiency | A | 15 | 50 | 300 | 17 | 20 | N | N | A |
| Mild iron deficiency | A | 13 | 30 | 350 | 8 | 10 | N | I | P |
| Severe iron deficiency | A | 7 | 20 | 450 | 4 | 4 | Microcytosis Hypochromia poikilocytosis | I | P |
| Acute inflammation | N | 15 | 50 | 280 | 15 | 300 | N | N or D | A |
| Chronic inflammation | I | 9 | 20 | 220 | 15 | 300 | Microcytosis hypochromia | N or D | A |

Abbreviations: N, normal; D, decreased; I, increased; P, present; A, absent

Iron deficiency also produces abnormalities in brain metabolism. There is now an important body of evidence showing delayed sensory development, motor function, and language skills in young children with iron deficiency. These do improve slowly with correction of iron deficiency, but children are still not back to normal some years later. Iron deficiency sometimes creates a desire to eat odd substances such as ice, clay, or starch, a disorder called "pica." This may result in young children chewing on painted surfaces, causing lead poisoning.

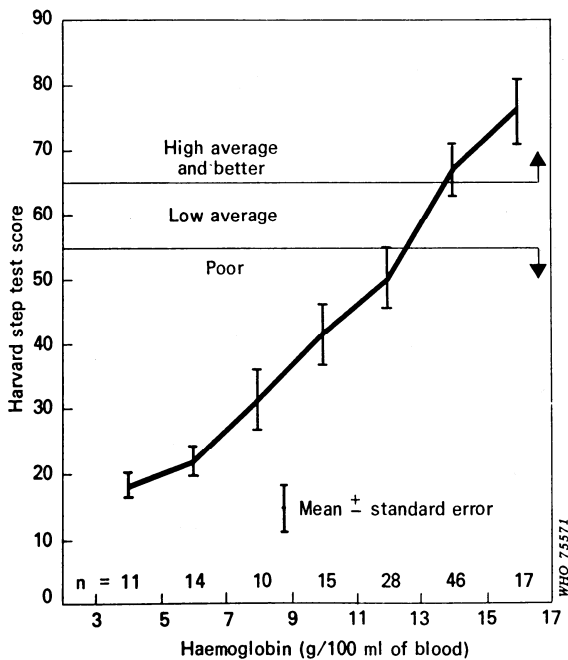
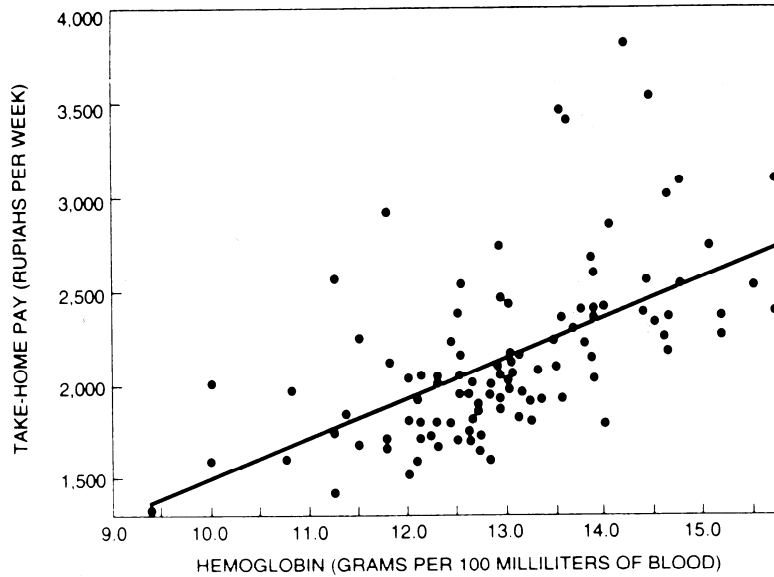


Figure 2.9 Exercise capacity in subjects with iron deficiency anemia. (From Viteri FE and Torún B, *Clin. Haematol.* 3: 609, 1974.)



PRODUCTIVITY of Indonesian rubber tappers varies with hemoglobin levels. The diagonal line marks the association between income and hemoglobin.

Figure 2.10 Take home pay as a function of hemoglobin concentration in Indonesian rubber workers. (From Scrimshaw NS. *Scientific American* pp.46-52, 1991.)

Treatment of Iron Deficiency

The treatment of iron deficiency consists of administration of oral or parenteral iron preparations when iron intake in the diet is inadequate to meet body needs. Oral iron usually corrects the deficiency just as rapidly and completely as parenteral iron if GI absorption is normal. Improvement in hemoglobin concentration typically begins within two weeks.

- Several therapeutic iron salts are available as supplements. Usual dosing is 600-1200 mg of an iron salt PO QD.
- Most common adverse effects are GI: constipation, abdominal pain, dyspepsia, stool discoloration, and nausea and vomiting.
- Adverse effects can diminish with use and may be reduced by taking iron immediately after meals for a few days.
- Liquid preparations of iron salts can produce tooth discoloration, a superficial and temporary staining of tooth enamel.

Parenteral iron is usually reserved for patients unable to tolerate or absorb oral iron:

1. Iron dextran: $\text{Fe}(\text{OH})_3$ and low molecular weight dextran containing 50 mg/mL elemental iron. Usual dosing is 20-40 mL in several hundred mL NS given as IV infusion over 1-2 hr
2. Iron sucrose: Polynuclear $\text{Fe}(\text{OH})_3$ complexed with sucrose. Usual dosing is 100-200 mg, given IV 1-3 times weekly.

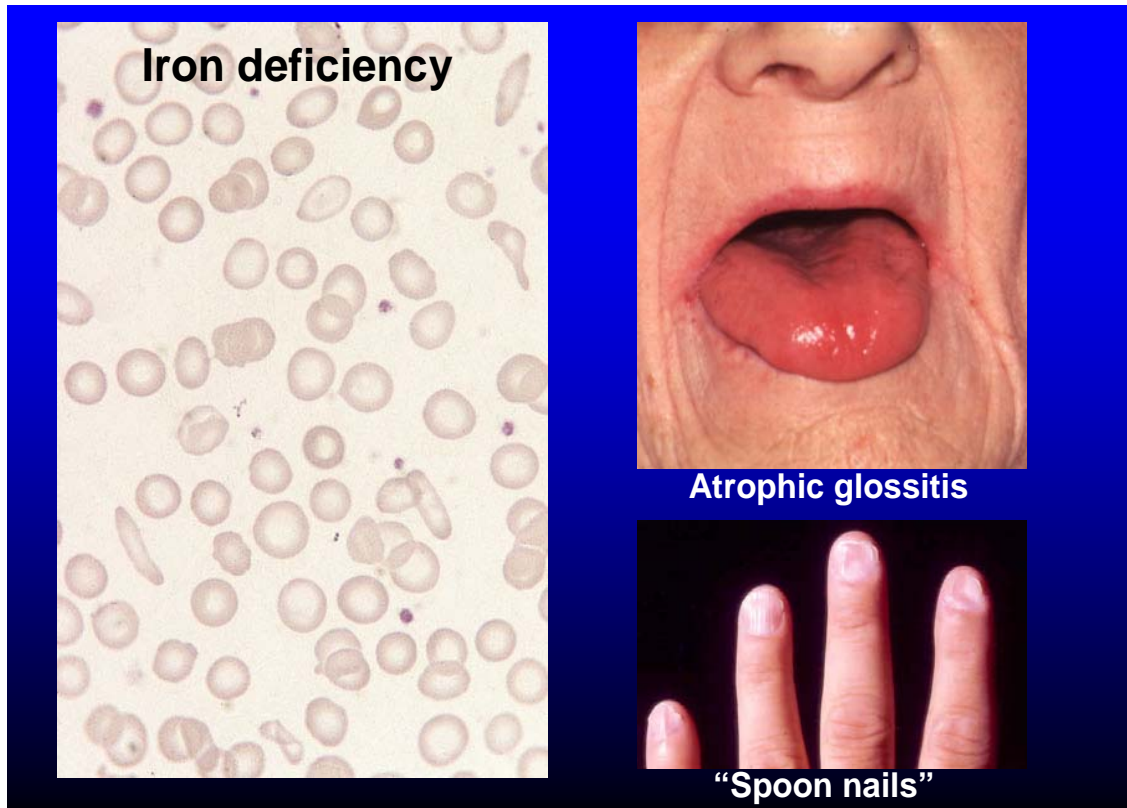


Figure 2.11 – Clinical Findings in Iron Deficiency

B. Anemia of Inflammation

Inflammation that lasts for weeks regularly leads to anemia. Usually the hematocrit is in the 25-32% range. While iron deficiency is the most common cause of anemia worldwide, **anemia of inflammation is the second most common cause and the most common type of anemia in hospitalized persons.** Inflammation may be due to infection, such as pneumonia, to an inflammatory disease like rheumatoid arthritis, or to a malignant tumor, even when symptoms of inflammation are not apparent.

The anemia of inflammation (aka, the anemia of chronic disease) has three pathophysiologic mechanisms

- **Sequestration of iron in macrophages, resulting in low plasma iron levels.**
- **Lower levels of erythropoietin than expected for the degree of anemia.**
- **Decreased marrow response to erythropoietin.**

All of these effects are due to the release of various cytokines in inflammatory states.

The most important of these mechanisms is the reduction in plasma iron, making less available for red cell production. Bacterial polysaccharides and the cytokine interleukin 6 generated during inflammation are powerful stimulators of hepcidin production by hepatocytes. Hepcidin in turn suppresses ferroportin and iron remains in macrophages (Fig. 2.8).

Both serum iron and transferrin levels (TIBC) fall, rapidly restricting the iron supply to marrow erythroid precursors and decreasing red cell production. Initially the anemia is normochromic and normocytic, but with prolonged inflammation, microcytosis develops. **In contrast to true iron deficiency anemia, in inflammation, storage iron as reflected in the serum ferritin is normal or elevated.** The drop in serum iron is thought to be beneficial to the host as it deprives invading bacteria of an essential growth factor. Hepcidin itself has bactericidal properties *in vitro* and may contribute to host defenses.

The optimal treatment of the anemia of inflammation is the elimination of the cause of the inflammation. It does not respond to iron therapy. It may improve with administration of erythropoietin.

To distinguish iron deficiency from the anemia of inflammation, both of which may be microcytic, the transferrin level (TIBC) and the ferritin are the most helpful tests. (see Table 2.4) Elevated markers of inflammation such as the sedimentation rate (ESR) or C-reactive protein (CRP) help confirm the presence of an inflammatory state.

Do not rely on the serum iron or the % saturation of transferrin. Both are low in both conditions.

Table 2.4: IRON DEFICIENCY VS ANEMIA OF INFLAMMATION

| | Serum Iron | TIBC | % Saturation | Ferritin |
|------------------------|------------|------|--------------|---------------|
| Iron deficiency | ↓ | ⊕ | ↓ | ⊖ (<30) |
| Anemia of inflammation | ↓ | ⊖ | ↓ | N or ⊕ (>100) |

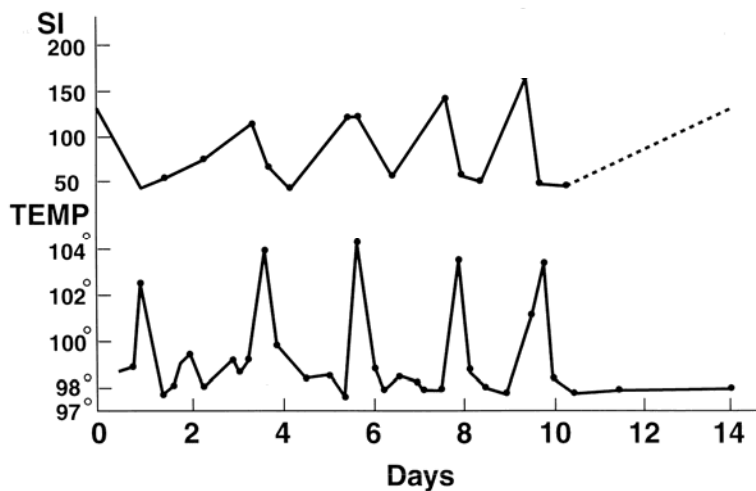


Figure 2.12. The effect of induced fever on plasma iron (PI) in a human subject. With each temperature elevation, the plasma iron drops sharply and returns to normal shortly after cessation of fever. (From Cartwright GE and Wintrobe MM, *Adv. Int. Med.* 5, 182, 1952.)

C. Low Erythropoietin Anemias

In addition to inflammation, a variety of chronic medical conditions can cause decreased erythropoietin production, which in turn causes a hypoproliferative anemia. The most important of these is chronic kidney disease, which is discussed below. Other examples of conditions that cause low-erythropoietin anemia include endocrine deficiency states and severe malnutrition.

D. Chronic Kidney Disease

Anemia usually appears when the creatinine clearance falls from the normal adult level of about 100 ml/min to about 25 ml/min, indicating a 75% loss of renal function. The severity of anemia correlates roughly with the degree of renal failure and is largely due to destruction of the renal erythropoietin-producing mechanism (Fig. 2.13). Red cell indices usually are normal. Young reticulocytes usually are not observed in the circulation, despite the severity of the anemia, because erythropoietin levels are depressed. Injections of recombinant erythropoietin dramatically improve anemia in patients with chronic renal failure. This treatment both eliminates the need for transfusions and improves the quality of life.

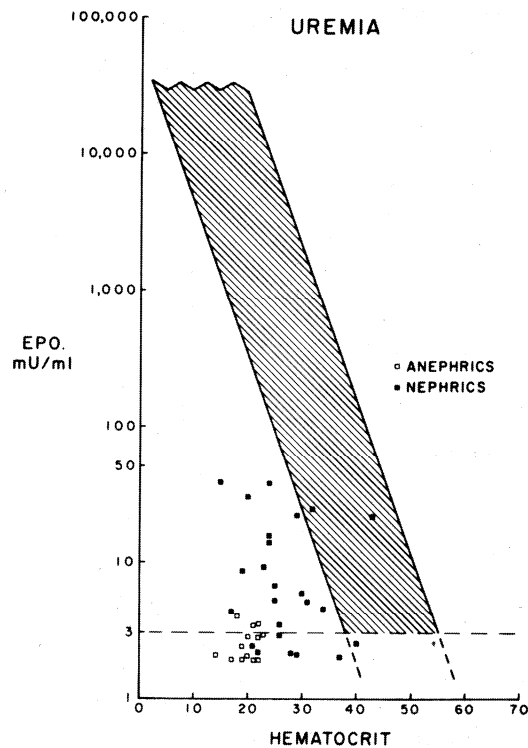


Figure 2.13. Relationship between hematocrit and plasma erythropoietin in patients with chronic renal failure, with and without kidneys. The cross-hatched area shows the normal range of values. (From Erslev, AJ, et al.: Plasma erythropoietin in health and disease. *Ann Clin Lab Sci* 10: 250, 1980.)

FYI

A rare but physiologically instructive cause of a low hematocrit is a rightward shift of the oxygen dissociation curve due to a hemoglobin mutation or to an increased level of erythrocyte 2,3-diphosphoglycerate (2,3 DPG). In this instance, the amount of oxygen released from blood with a right-shifted oxygen dissociation curve for a given drop in PO_2 is greater than normal. As a result, the kidney needs to generate less erythropoietin to maintain its own oxygen tension in the normal range.

Mild right shifts of the oxygen dissociation curve also are observed in children. This is due to higher levels of 2,3-DPG in red cells which in turn is a consequence of higher serum phosphate levels. This right shift is one reason that the hematocrit is lower in children than in adults.

E. Anemias Due to Marrow Damage

Aplastic anemia

Aplastic anemia is a heterogeneous group of conditions in which the marrow is severely hypocellular. By definition, the red cell series plus one or more additional cell lines—i.e., granulocytes and/or megakaryocytes—is involved. The diagnosis of aplastic anemia is made from the combination of low hematocrit and white cell count or platelet count and markedly reduced cellularity on bone marrow exam. As in other hypoproliferative anemias, the reticulocyte count is low for the degree of anemia. The serum iron is elevated because of the marked decrease or absence of erythroid precursors to take up iron from transferrin. For more details see Chapter 1.

IV. Iron Overload

The body has no excretory pathway for iron. If body iron stores accumulate to the 15-20 gram range (normal is 1 gram) for any of the reasons discussed below, tissue damage occurs. When the stores exceed the sequestration capacity of the protective storage protein ferritin, iron exists in a reactive form causing tissue injury, probably by generating free radicals. **The most commonly affected organs are the liver (cirrhosis and liver cancer), the pancreas (diabetes), and the heart (congestive heart failure).** Arthritis, a variety of endocrine disorders including gonadal failure with impotence, and a peculiar bronze skin color complete the clinical picture.

Hereditary hemochromatosis is a common and important cause of iron overload. Early recognition and removal of iron prophylactically will prevent all the life-threatening complications. **It is most often caused by a mutation in the HFE gene on chromosome 6, resulting in the substitution of tyrosine for cysteine at position 282 of the HFE protein (Cys 282 tyr).** This mutation appeared in a Celtic or Viking ancestor about 2,000 years ago somewhere in Northwest Europe. As its ill effects are manifest only after the reproductive period, and it might have had some survival advantage by preventing iron deficiency anemia after blood loss, the mutation spread with the migrating population. **About 10% of all Caucasians are heterozygotes and 0.5% (1 in 200) are homozygotes. Only homozygotes develop symptomatic disease and penetrance is quite variable.** This particular mutation is uncommon or nonexistent in non-Caucasians and women are relatively spared, likely due to iron losses through menstruation or pregnancy.

A. Pathophysiology

The normal HFE protein is expressed on the surface of cells in a complex with $\beta 2$ microglobulin and the transferrin receptor. When HFE is mutated, this association does not occur. Recent evidence suggests that the failure of these proteins to associate leads to a **failure of hepcidin secretion by the liver.** Thus ferroportin continues to release iron to the plasma from duodenal enterocytes and macrophages despite very high plasma iron and ferritin levels. **In effect, the liver always behaves as though the patient is iron deficient.** Starting at birth, the small increase in iron absorption from a normal value of 1 mg to 2-5 mg daily may result in accumulations of 25-50 grams by about age 50. At that time, clinical manifestations—notably bronze skin, liver failure, and diabetes—usually become evident. The term “bronze diabetes” has long been applied to this disorder.

B. Lab Findings

The earliest abnormality is an increase in transferrin saturation. When a patient is found to have a saturation exceeding 45-50%, ferritin levels and genetic testing for the HFE gene are obtained. If hemochromatosis is detected when ferritin levels are less than 1,000 ng/ml, tissue damage is unlikely. Treatment is weekly phlebotomy of 500 cc of whole blood, thus removing about 250 mg of iron each time. It may take up to two years to deplete iron stores, after which 3 to 6 phlebotomies per year will prevent iron reaccumulation. Once tissue damage occurs, it is usually irreversible, though progression is slowed by treatment. All first degree relatives of the patient should have genetic counseling and testing so phlebotomy can be undertaken early and complications prevented (Fig. 2.14)

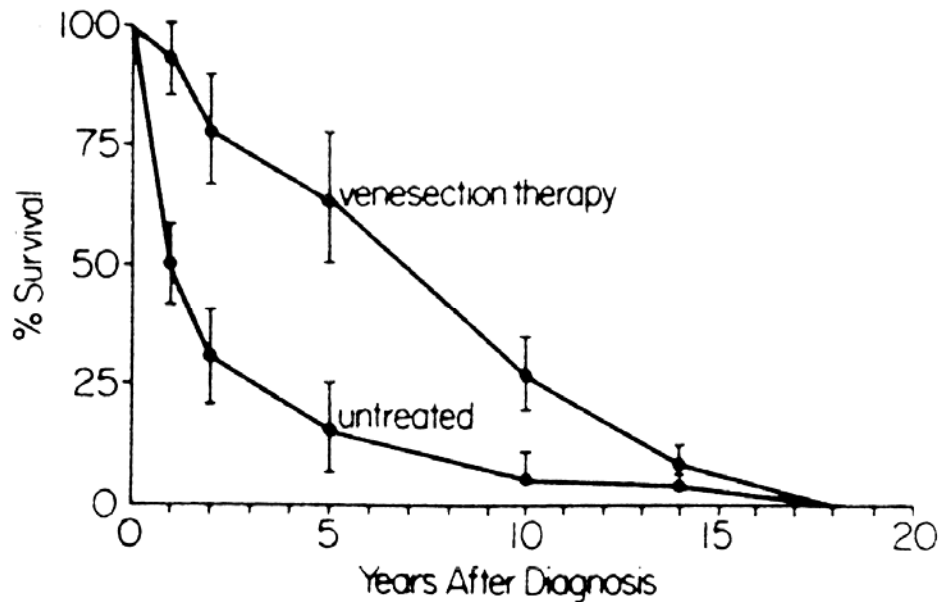


Figure 2.14. Life-table survival curves after diagnosis in phlebotomy treated and untreated groups of patients with idiopathic hemochromatosis. The vertical lines at each time interval represent ± 1 SE (From Bothwell, TH, et al., *Iron Metabolism in Man*. Oxford, Blackwell, 1979.)

Iron overload is seen in a number of other settings. Increased iron absorption occurs in chronic anemias that are due to ineffective erythropoiesis (thalassemia) or hemolysis. This is because anemia *per se* and increased marrow erythroid activity for any reason decrease hepcidin release from the liver. Iron overload is a very serious clinical problem in thalassemia major where repeated transfusions add to the iron burden. This is discussed further in Chapter 5.

Many patients with marrow disorders that cause a chronic hypoproliferative anemia (myelodysplasia, aplastic anemia) may need repeated red cell transfusions. As the recycled iron from the senescent transfused red cells cannot be excreted, **symptomatic iron overload occurs after about 100 units of blood** (250 mg iron/unit x 100 = 25 grams). Treatment with iron chelators can be used to decrease iron accumulation.

Acute iron poisoning is a preventable cause of death in young children. Iron tablets may resemble candy (M&Ms), and as few as three tablets could cause major toxicity. The gastrointestinal mucosa undergoes necrosis, leading to nausea, vomiting, and bloody diarrhea. Shock and coma follow. Emergent removal of iron from the GI tract and administration of the iron chelator deferoxamine have improved the survival of these gravely sick children.

V. Summary

Iron is essential to life but paradoxically cannot be free in the body because of its toxicity. Elegant methods are employed by the body to conserve iron and to shield it within transport and storage proteins. Iron deficiency is a world-wide problem that is easily recognized and treated. It is common in young children and in women in the child-bearing years as a result of an imbalance between supply and demand, whereas in older women and men it is commonly a result of gastrointestinal losses, of which cancer is the greatest concern. Early treatment of hemochromatosis will prevent all complications.

CHAPTER 3

VITAMIN B₁₂ (COBALAMIN) AND FOLIC ACID (FOLATE)

Key Concepts:

- Definition and morphologic features of megaloblastic anemia
- Vitamin B₁₂ deficiency
- Folate deficiency

Learning Objectives:

1. Describe, and be able to recognize under the microscope, the morphologic findings in the blood and bone marrow in megaloblastic anemia.
2. Describe the pathophysiology and the clinical and laboratory features of vitamin B₁₂ and folate deficiency, including the important similarities and differences between them.

I. Introduction

A key event in a rapidly dividing tissue such as the bone marrow is the synthesis of new DNA. Any process that inhibits DNA synthesis blocks production of all replicating cell lines. In the bone marrow, a block in DNA synthesis results in a nucleus that is immature for the degree of maturation in the cytoplasm. Red cell precursors with this abnormality are called megaloblasts rather than normoblasts. Deficiency of either folic acid or vitamin B₁₂ (also called cobalamin) causes megaloblastic anemia because it leads to a shortage of thymidine, which in turn leads to retarded DNA synthesis. Circulating red cells in megaloblastic anemia are typically larger than normal and are therefore called macrocytes.

II. Definitions

Macrocytic anemia is a subset of anemia in which the non-nucleated erythrocytes are larger than 100 femtoliters (fl). It is found in association with liver disease, alcoholism, hypothyroidism, and several forms of marrow damage as well as in B₁₂ and folic acid deficiency. Macrocytes are red cells released before they have divided enough times to be normal-sized. There are two mechanisms for decreased divisions:

- early release forced by demand for new red cells
- retarded DNA synthesis.

For example, because reticulocytes are considerably larger than mature red cells (some young ones may be 150 fl), hemolytic anemia with a high reticulocyte count may be macrocytic on that basis alone. In megaloblastic anemia the red cells are macrocytic, presumably because retarded DNA synthesis has reduced the number of cell divisions that normally occur as the cytoplasm matures.

Megaloblastic anemia is a specific subset of the general class of macrocytic anemias. Megaloblastosis is the visible change in nucleated cells that results from a lag in nuclear maturation relative to cytoplasmic maturation. It is the morphologic counterpart of reduced DNA:RNA and thymine:uracil ratios noted in biochemical assays of megaloblastic marrows.

Folic acid deficiency is probably the most common cause of megaloblastic anemia in the general population, but cobalamin deficiency may be a more common cause in parts of the world where intake of animal protein, the dietary source of vitamin B₁₂, is low. **Megaloblastic anemia due to vitamin deficiency is a manifestation of advanced deficiency.** In a referral hospital with a large proportion of cancer patients, however, the most common cause of megaloblastic change is cancer chemotherapy. Megaloblastosis is seen commonly after chemotherapy with methotrexate. Marked macrocytosis and hypersegmentation of neutrophils occur in patients treated with hydroxyurea. Table 3.1 gives other examples.

Table 3.1

**DRUGS ASSOCIATED WITH MEGALOBLASTOSIS:
CLASSES AND EXAMPLES**

| | |
|-------------------------------------|---|
| Megaloblastosis Common | Dihydrofolate reductase inhibitors: methotrexate Purine analogs: 6 mercaptopurine, 6-thioguanine Pyrimidine analogs: 5 fluorouracil, zidovudine (AZT) Ribonucleotide reductase inhibitors: hydroxyurea |
| Megaloblastosis Uncommon | Anticonvulsants: phenytoin Oxidants: nitrous oxide |

Megaloblastosis is best seen in the erythroid cell.

- Instead of having a small, compact nucleus, megaloblasts at later stages of maturation still have a large nucleus with finely dispersed chromatin, much younger-looking than expected for the degree of hemoglobinization of the cytoplasm.
- A second feature is the presence of giant bands in the bone marrow.
- A third feature is hypersegmentation of the neutrophils in the peripheral blood. In normal people, most neutrophils will have two, three or four lobes, and fewer than five percent will have five lobes. Neutrophils with six or more lobes are seen in megaloblastosis.

The peripheral blood expressions of megaloblastosis (macrocytosis and neutrophil hypersegmentation) may occur with minimal anemia. Hypersegmentation of neutrophils should lead to a search for the cause—most likely B₁₂ or folate deficiency or some drug known to cause megaloblastosis.

III. Deficiency of Folate or Vitamin B₁₂

Vitamin deficiency is almost invariably the result of one or more of the following five processes:

- **Inadequate intake** of folic acid is common among alcoholics and institutionalized patients. Strict vegetarians ingest very little vitamin B₁₂ and should take a vitamin pill containing B₁₂. Other highly restricted diets lacking in meat and fresh vegetables may produce folic acid deficiency.
- **Malabsorption** of vitamin B₁₂ may be due to a lack of intrinsic factor. Malabsorption of folate may occur in celiac disease. Drugs may prevent removal of glutamic acid residues on folic acid and thereby impair its absorption.
- **Increased utilization or loss.** Pregnancy and hemolysis increase the need for folate by accelerating its rate of use and are also extremely rare causes of vitamin B₁₂ deficiency.
- **Drug inhibition** of the physiologic function of the vitamin. For example, methotrexate is a folic acid antagonist. Nitrous oxide inactivates some of the cobalamin, and may be hazardous in subjects with marginal stores.
- **Genetic defects** of transcobalamin II are rare congenital disorders of B₁₂ metabolism.

IV. Vitamin B₁₂, the Red Vitamin

FYI

History

The concept of ill health due to inadequate intake of food is as old as mankind, but the concept of diseases due to specific deficiencies was slow to gain recognition in the medical world. Two centuries ago, a Scottish naval surgeon, James Lind, proved that fresh lemons and limes cured and prevented scurvy among sailors, but the next clear proof of a specific disease due to a specific nutritional deficiency was not recognized until the early 20th century, when thiamine deficiency was shown to cause beriberi among rice-eating peoples of Southeast Asia.

Pernicious anemia was well described morphologically and clinically for at least half a century before it was shown to be caused by a nutritional deficiency, although the distinguished American physician, Austin Flint, wrote in 1860 that the disorder was probably due to a failure to assimilate some necessary nutrient from the diet. Dr. Flint also proposed to accept the credit for his idea as soon as someone could do the work necessary to prove its validity!

Unfortunately, he did not live long enough to see the proof offered by Minot and Murphy in 1926.

During and immediately after World War I, George Whipple and Frieda Robscheit-Robbins studied the anemia induced in dogs when blood was removed each day. Their work was intended to determine the most efficacious diet for the regeneration of blood, and they found, of course, that refeeding the blood to the dog was most efficacious. The next most effective diet was one that was high in liver. George Minot, an investigative physician in Boston, knew of the work of Whipple and Robscheit-Robbins and was of the opinion that pernicious anemia might be a special kind of nutritional deficiency. Minot's view was contrary to the current dogma, since patients with pernicious anemia usually did not appear undernourished and in fact, were sometimes moderately obese. But when Minot and Murphy fed a half pound of lightly cooked liver (!) each day to patients with pernicious anemia, the patients showed a remarkable hematologic improvement. Minot and Murphy's reward for their documented and confirmed observation was international acclaim and a Nobel Prize. Thus, it had been established as early as 1926 that some substance in liver was curative for patients with pernicious anemia. A liver extract, to be injected, was prepared soon afterward. The injection of liver extract every two to four weeks prevented death and neurologic disease, and corrected anemia in patients with pernicious anemia.

For several decades before the therapeutic triumph of Minot and Murphy, it was known that patients with pernicious anemia had severe atrophy of the gastric mucosa. Also, their gastric juices were known to be scanty and lacking in acid and peptic activity. William Castle postulated that the gastric pathology might be playing a causal role in pernicious anemia, and he proved his postulate with brilliantly conceived and controlled therapeutic trials. Feeding about 0.25 kg of hamburger to a patient daily for ten days failed to improve the reticulocyte count or anemia. During the next ten days, however, he fed the patient the same quantity of hamburger with normal human gastric juice, and generated an impressive reticulocyte response, increase in hemoglobin and sense of well-being. We now know that the function of gastric "intrinsic factor"--a phrase coined by Castle--is to bind dietary B₁₂ (found in the hamburger in this experiment) and facilitate its absorption in the ileum.

The identification of the anti-pernicious-anemia principle in liver had to wait until 1948. In that year, scientists at American and British pharmaceutical firms simultaneously and independently reported the isolation and crystallization of vitamin B₁₂. Each of these groups had demonstrated that the crystallized vitamin was extremely effective in the treatment of pernicious anemia. The first reports showed that, on one occasion, the injection of as little as ten micrograms of vitamin B₁₂ led to a significant hematologic improvement. Vitamin B₁₂ was thus the most potent vitamin known at that time.

It was soon demonstrated that the red vitamin (so named because of its deep red color) contained cobalt bound into a tetrapyrrole ring, somewhat analogous to the iron in heme. A second Nobel prize related to vitamin B₁₂ was awarded to Dorothy Hodgkin for her X-ray crystallographic studies demonstrating the exact structure of the vitamin (molecular weight 1,350 daltons), as diagrammed in Figure 3.1. Commercial vitamin B₁₂ is derived from microbial synthesis, an efficient and inexpensive process compared to chemical synthesis; a quantity sufficient to treat a pernicious anemia patient for a whole year costs no more than a few dollars.

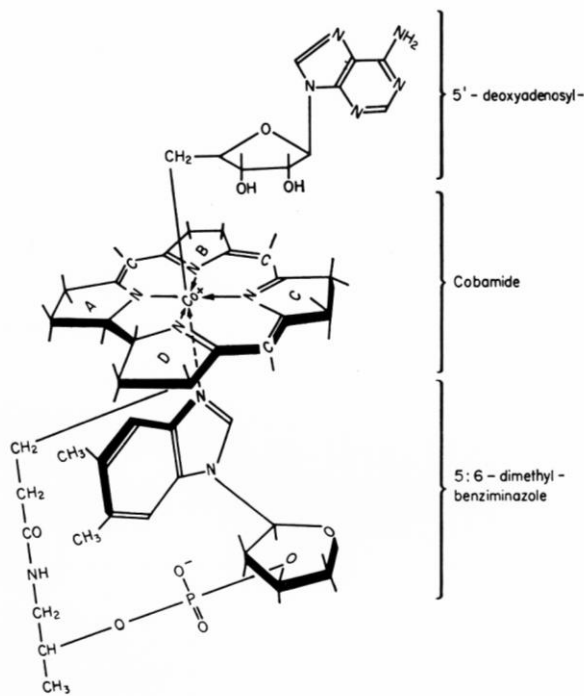


Figure 3.1. Structural formula of deoxyadenosyl cobalamin. Cyanocobalamin has a CN group ligated to the cobalt in a place of deoxyadenosyl, and methylcobalamin would have a methyl group at that locus.

A. Physiology

The typical daily Western diet contains five to thirty micrograms (μg) of vitamin B₁₂ in animal, bird and fish products, of which the liver and kidney are especially rich sources. Peptic digestion contributes to the freeing of B₁₂ from binding proteins in food. Two to five μg of vitamin B₁₂ are absorbed daily.

- In the stomach, some of the B₁₂ complexes with **R binders**. "R binders" are related to transcobalamins I and III, which are B₁₂-binding proteins found in serum. Their function in the gut is unknown but it has been speculated that they help dispose of inactive cobalamin analogues.
- In the duodenum, B₁₂ is released from the R binders by pancreatic digestion and binds to **intrinsic factor. Intrinsic factor (IF) is a labile glycoprotein secreted by gastric parietal cells. B₁₂ requires IF for absorption.** When complexed with B₁₂, IF is protected from degradation by enzymes in the small intestine.
- **The IF-B₁₂ complex is adsorbed to brush border receptors in the ileum,** and the vitamin enters the circulation via the ileal cells. In the absence of intrinsic factor, only one to two percent of B₁₂ is absorbed, but with intrinsic factor, 60-90% of a test dose of aqueous B₁₂ will be absorbed.
- From the ileum, B₁₂ is carried by **transcobalamin II** to the liver, where some of the vitamin is stored.

The human body ordinarily contains 2,000 to 5,000 μg of vitamin B₁₂. Approximately 0.1% to 0.2% of the body store is lost daily, so **the biologic half-life of the vitamin is approximately a year and a half, and clinical deficiency becomes apparent only after several years of grossly inadequate absorption.** It is likely that megaloblastic anemia and neurologic disease due to vitamin B₁₂ deficiency occur after body stores of the vitamin have decreased to less than 20% of normal.

B. Biochemistry

Cyanocobalamin, a stable form of vitamin B₁₂ was the first cobalt-containing organic compound shown to have a biologic role. Students of animal husbandry knew that cobalt was an essential nutrient for sheep. After isolation, crystallization and partial characterization of cyanocobalamin from liver in 1948, the role of cobalt in the maintenance of life of all animals and many bacteria began to emerge. Plants do not require or synthesize vitamin B₁₂.

The mechanism by which B₁₂ deficiency leads to relative failure of DNA synthesis is not understood in humans because no B₁₂-dependent ribonucleotide reductase has been described in mammalian cells. In mammals, only two reactions clearly requiring vitamin B₁₂ have been identified.

1. The reversible conversion of methylmalonyl CoA to succinyl CoA is catalyzed by methylmalonyl CoA mutase, which requires adenosylcobalamin as a cofactor (Fig. 3.2). Deficient activity of methylmalonyl CoA mutase is thought by some investigators to be the cause of the neurologic disease in B₁₂ deficiency.

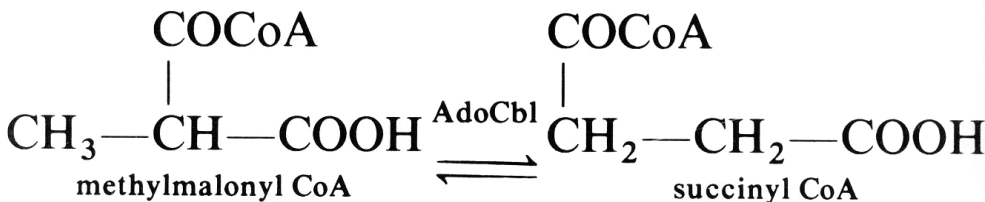


Figure 3.2. Conversion of methylmalonyl CoA to succinyl CoA by adenosylcobalamin.

2. The methylation of homocysteine to form methionine requires enzyme-bound methylcobalamin and N⁵-methyltetrahydrofolate as a methyl donor (Fig. 3.3). Methionine provides a methyl group for modification of myelin basic protein; a deficiency of methionine is believed by some to decrease synthesis of myelin, leading to demyelination of the posterior and lateral columns of the spinal cord. However, since homocysteine accumulation in serum is regularly seen in folate deficiency as well as in B₁₂ deficiency, the failure of this enzyme reaction does not explain the extreme rarity of neurologic disease in folate deficiency and the frequency of neurologic disease in cobalamin deficiency.

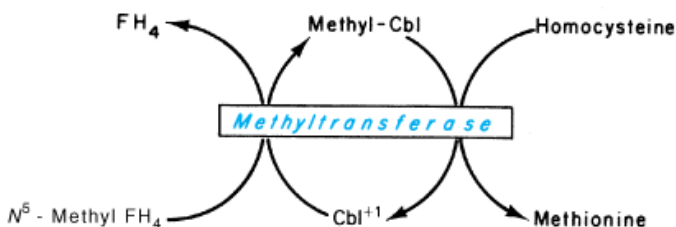


Figure 3.3. Linkage of folate and cobalamin in the methylation of homocysteine to form methionine. (*FH₄*, tetrahydrofolate; *Cbl*, cobalamin)

Source: Lichtman MA, Beutler E, Kipps TJ, Seligsohn U, Kaushansky K, Prchal JT: *Williams Hematology*, 7th Edition: <http://www.accessmedicine.com>
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The enzyme-bound cobalamin serves as a methyl transferase. The interdependence of vitamin B₁₂ and folate in this methyl transfer is shown schematically in Figure 3.3, which indicates that the methyl group of methylcobalamin is from methyltetrahydrofolate (abbreviated in Figure 3.3 as N⁵-methyl FH₄). There is evidence that the sera of vitamin B₁₂-deficient patients contain higher than expected levels of methyltetrahydrofolate. This "trapping" of folate in the serum as methyltetrahydrofolate is thought to result in an intracellular deficit of a tetrahydrofolate needed as a cofactor for thymidylate synthetase, which in turn is essential for DNA synthesis. Thus, **it appears that B₁₂ deficiency leads to a functional folate deficiency in dividing cells.** If intracellular folate deficiency is the common lesion in B₁₂ and folate deficiency, the morphologic identity of the megaloblastic abnormalities in these two conditions is understandable.

Levels of serum homocysteine and methylmalonate (MMA) rise in B₁₂ deficiency. It should be noted that **homocysteine will accumulate in the plasma in either folate or B₁₂ deficiency, but MMA accumulates only in B₁₂ deficiency, not folate deficiency.** Measurement of MMA may be a useful test in the evaluation of unexplained peripheral neuropathy, especially in patients who have no hematologic clues of megaloblastosis.

C. Mechanisms of Vitamin B₁₂ Deficiency

The most common cause of B₁₂ deficiency in the Western world is an acquired loss of intrinsic factor, also known as "pernicious anemia." In humans, intrinsic factor is secreted only by gastric parietal cells. Parietal cells may be lost by autoimmune destruction of the gastric mucosa, by physical or chemical injury, or by surgical removal. Autoimmune destruction of gastric parietal cells is far and away the most common cause of B₁₂ deficiency.

The risk of vitamin B₁₂ deficiency from inadequate intake is greatest in strict vegans, who eat neither eggs nor milk products. There are few true vegans in the United States, so dietary insufficiency is rarely a cause of megaloblastic anemia. Strict vegans should be advised to take pills containing vitamin B₁₂ and folate.

Because B₁₂ is avidly taken up and biochemically altered by many microorganisms, **one might expect some patients with small intestinal disease accompanied by massive bacterial overgrowth to suffer from B₁₂ malabsorption and deficiency.** This is the case in several anatomic and physiologic disorders of the gut, especially large diverticula of the small intestine or a surgically created blind loop (hence the term "blind loop syndrome"). Anaerobic organisms such as *Bacteroides* avidly bind vitamin B₁₂, even when it is coupled to intrinsic factor,

rendering the vitamin unavailable for absorption. Fish tapeworm infestation is common in Finland, and B₁₂ deficiency often follows because the worm is a successful competitor for B₁₂.

The pancreas also plays a role in B₁₂ absorption; patients with extensive chronic pancreatitis or with cystic fibrosis often fail to absorb B₁₂ when given an oral test dose. The malabsorption is corrected by the simultaneous oral administration of pancreatic extract or trypsin. Clinical B₁₂ deficiency, however, is very rare in those patients.

At the ileum, B₁₂ is transferred from its intrinsic factor complex into the circulation and cells of the body. **Patients who have lost their ileal function through surgical removal or bypass, or because of extensive inflammatory destruction of the mucosa, as in regional ileitis (Crohn's disease), may become deficient because of a failure to absorb vitamin B₁₂.** Thus, structural injury to the gastrointestinal tract (destruction of the gastric mucosa, injury to the pancreas, blind loop or giant diverticulae of the small intestine, or destruction of the ileum) leads to B₁₂ deficiency more commonly than dietary nutritional deficiency or genetic metabolic derangement.

FYI

A genetic deficiency of transcobalamin II is associated with severe megaloblastic anemia early in life.

Nitrous oxide ("laughing gas") is a widely used anesthetic. The use of nitrous oxide for anesthesia in unrecognized subclinical B₁₂ deficiency has led to severe neuropathy. Recreational abuse of nitrous oxide has led to macrocytosis and neuropathy indistinguishable from B₁₂ deficiency. Nitrous oxide reduces the activity of methionine synthetase probably by inactivating the B₁₂ coenzyme portion of the molecule. Nitrous oxide also destroys methyl cobalamin. This appears to be a new mechanism for developing megaloblastic anemia. It responds to treatment with vitamin B₁₂ as expected.

D. Pernicious Anemia

A formal definition of pernicious anemia is autoimmune or idiopathic gastric atrophy sufficient to cause vitamin B₁₂ malabsorption due to lack of intrinsic factor.

1. Serology

Sera from most patients with pernicious anemia contain autoantibodies to gastric parietal cells, but these antibodies have low specificity. Anti-parietal cell antibodies are relatively common in patients with other autoimmune diseases such as myxedema, diabetes, and gastritis. **Autoantibodies to intrinsic factor are present in only about 60% to 70% of sera from patients with pernicious anemia, but they have high specificity.** The presence of intrinsic factor antibodies is almost diagnostic of "pernicious anemia." **Achlorhydria, achylia, and gastric atrophy are not corrected by the administration of vitamin B₁₂ because they are the cause, not the result of vitamin deficiency. The failure to absorb vitamin B₁₂ is permanent in patients with pernicious anemia, who must receive supplemental B₁₂ for life.**

2. Clinical Manifestations

"Pernicious" anemia is now a misnomer, because the disease is simple to treat with an injection of vitamin B₁₂. Vitamin B₁₂ deficiency is a disease of the second half of life, the incidence being roughly one new case per year per 4,000 people over 40 years of age. There also is a hereditary form of lack of functional intrinsic factor, known as "juvenile pernicious anemia." This rare condition is inherited as a recessive trait, resulting in non-functional intrinsic factor.

In addition to the usual physical findings of anemia, the pernicious anemia patient may complain of a **sore tongue**, which is often smooth, red, and glistening due to lack of papillae. The tongue symptoms and appearance will improve promptly after the correction of the vitamin B₁₂ deficiency. **Patients may complain of paresthesias and difficulty walking in the dark**, and may appear neurotic. **Neurologic examination will often reveal absence of vibratory sensation and proprioception.** In advanced stages, the neurologic aspects of vitamin B₁₂ deficiency may cause spastic paralysis of lower extremities, loss of sphincter control, and dementia—a severe derangement of thought processes sometimes called "megaloblastic madness."

Vitamin B₁₂ deficiency leads to demyelination of lateral and dorsal spinal cord tracts (Fig. 3.4). Individuals with mild to moderate neurologic deficits can be expected to make a complete recovery following B₁₂ therapy, though the time required to recover full function may be as much as twelve to sixteen months. **Recovery from advanced neurologic damage (inability to walk, incontinence) is unlikely to be complete.** **Neurologic disease in a person with megaloblastic anemia is highly suggestive of B₁₂ deficiency rather than folate deficiency.** In some patients, the neurologic disease is prominent with little or no anemia, while others have prominent anemia with normal neurologic examinations. **If a patient with B₁₂ deficiency is mistakenly treated with folate, neurologic disease may progress to irreversible crippling.**

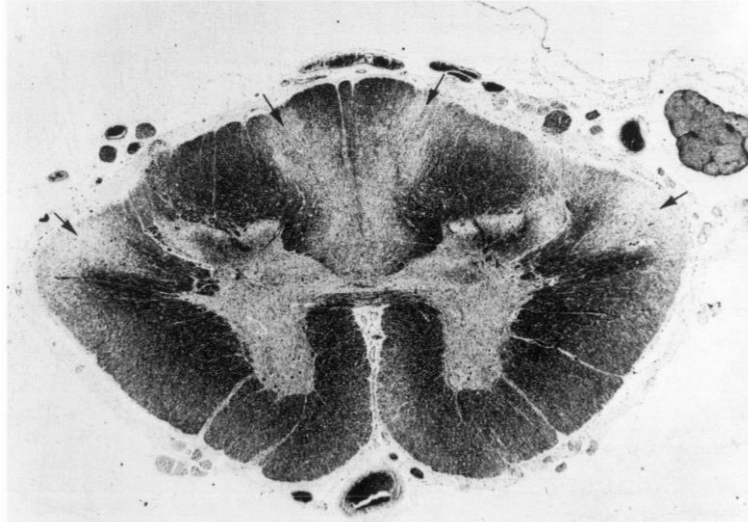


Figure 3.4. Degeneration of the posterior and lateral columns of the spinal cord in vitamin B₁₂ deficiency. The arrows point to areas of demyelination and loss of nerve fibers. (From Kass, LS: *Pernicious Anemia*. Phila., WB Saunders Co., 1976.)

E. Cell Production

Since vitamin B₁₂ and folate are essential to DNA synthesis, one would expect all multiplying cells to be affected with analogous biochemical and morphological defects. Megaloblastosis is most easily recognized in erythroid and myeloid cells. **Pancytopenia (decreased red cell, platelet, and granulocyte production) may occur in severe deficiency.** Macrocytosis and dissociation of maturation in nucleus and cytoplasm have also been described in cells from other rapidly growing tissues such as skin, tongue, testis, bronchus, stomach, and cervix. The fetus and newborn also have special need for B₁₂. There are cases of infants who became B₁₂ deficient while nursing from mothers who were strict vegans or who had gastric bypass for obesity. It is prudent to give B₁₂ supplements to such mothers.

The kinetic classification of megaloblastic anemia is ineffective erythropoiesis. Red cell destruction is mainly in the marrow rather than in the blood (see chapter 2). The erythroid marrow is hypercellular with a 1:1 G:E ratio, and the total marrow mass is greatly increased. The serum bilirubin and serum lactic dehydrogenase (LDH) may be increased as one would expect in red cell destruction, but the absolute reticulocyte count is decreased because few new cells are released.

F. The Schilling Test

The most common cause of B₁₂ deficiency is malabsorption rather than dietary deficiency. There are several different mechanisms of B₁₂ malabsorption, and a radioactive B₁₂ absorption study is a procedure to sort them out. Though no longer used clinically, it elucidates the pathophysiology of B₁₂ deficiency.

The urine radioactivity test (Schilling Test) was developed to measure B₁₂ absorption. Radioactivity appearing in the urine after an oral dose of radioactive B₁₂ represents absorbed vitamin. Because absorbed B₁₂ is normally bound to plasma transcobalamins, no radioactive vitamin is filtered at the glomerulus (molecular weight of B₁₂ is 1,350 daltons). However, if one injects a large quantity (1,000 µg) of nonradioactive vitamin B₁₂ two hours after the oral dose, the transcobalamins will be saturated, much of the absorbed radioactive B₁₂ will be unbound, and about a third of the absorbed radioactive vitamin will normally appear in urine in the next 24 hours.

Serum B₁₂ concentration should be measured in all patients with dementia and neuropathy, so that this treatable cause of serious neurologic disease is not overlooked. Measurements of serum homocysteine and methylmalonate are useful in defining metabolic evidence of cobalamin deficiency. One should bear in mind that as tests for clinically significant cobalamin deficiency, these measurements appear to have excellent sensitivity but poor specificity. Many subjects with elevated serum levels of these metabolites don't have and never develop clinical evidence of cobalamin deficiency.

G. Treatment of B-12 deficiency

- Vitamin B₁₂ is used to treat or prevent deficiency, usually given parenterally. Available in oral and parenteral forms, including intranasal gel and spray forms.
- Available as cyanocobalamin or hydroxycobalamin. Usually dosed at 100-1000 µg IM QD or Q2D for 1-2 weeks, then 100-1000 µg IM Q month for life.

- Essentially nontoxic, even in large doses.
- Bone marrow function usually returns to normal within 48 hr, reticulocytosis begins on 2nd or 3rd day and is maximal by 5-10 days. Hemoglobin begins to increase by the first week and is normal in 1-2 months.

V. Folic Acid

A. History

Within a few years after the demonstration of the remarkable efficacy of liver in the treatment of pernicious anemia, it was realized that not all megaloblastic anemia was due to a deficiency of the factor present in injectable liver extract.

FYI

In the 1930s Lucy Wills, a young physician from London, went to Bombay to study the megaloblastic anemia that was common in late pregnancy in India. She demonstrated in a convincing manner that injections of purified liver extract (so wonderfully efficacious in pernicious anemia in London) did not benefit the megaloblastic anemia of pregnancy in Bombay. She did find that eating generous amounts of "marmite" (a yeast extract) led to impressive hematologic and subjective improvement. From this observation came the term "Wills Factor" to identify that beneficial nutrient in yeast. In 1946 folic acid was identified and synthesized by scientists at Lederle Laboratories studying growth factors for certain bacteria. It soon became apparent that Wills Factor was folic acid.

B. Metabolism and Biochemistry

Folic acid (Figure 3.5) is a small molecule (molecular weight 440) that is absorbed in the small intestine, especially in the jejunum. Both passive diffusion and active transport have been demonstrated. Yeast is the richest nonmedicinal source of folate, but many vegetables, dairy products, and seafoods are excellent dietary sources. Food folate found in spinach, beans, broccoli, and other green leafy vegetables is in the polyglutamate form. An intestinal brush border deconjugase cleaves all but the last glutamate and thus enhances absorption. The daily food intake of folate is 200 to 400 μg and the daily nutritional requirement is about 50 μg . The vitamin is stored in the liver and other cells. **The biologic half-life of folic acid is less than one month, in contrast to the 18 month half-life of vitamin B₁₂.**

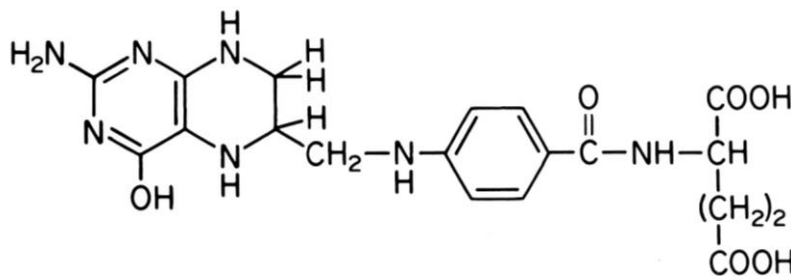


Figure 3.5. Chemical structure of folic acid (pteroylglutamic acid.).

Folate coenzymes transfer single carbon units in various oxidation states, such as methyl, formyl and formate, in a large variety of essential processes. For example, the methylation of deoxyuridine monophosphate to deoxythymidine monophosphate is catalyzed by thymidylate synthetase in a reaction linked to dihydrofolate reductase. The presence of these enzymes has been demonstrated in mammalian bone marrow. Other reactions requiring folic acid coenzymes are serine conversion to glycine, histidine catabolism, methionine synthesis from homocysteine, and purine synthesis.

C. Mechanisms of Deficiency

1. Inadequate diet

Diets composed exclusively of tea and toast or brandy and beer rapidly lead to folate deficiency. Food folate can be lost because it is easily oxidized when vegetables are cooked in boiling water. One physician investigator intentionally induced folic acid deficiency in himself in three months merely by thoroughly boiling his food and discarding the water.

2. Malabsorption

Serious decrements in folate absorption occur in celiac disease, probably due to defective active transport or lack of the deconjugase.

3. Increased utilization or loss

Increased metabolic requirement and accelerated turnover of folate explain the folic acid deficiencies in **pregnancy** and **chronic hemolytic anemia**. **The incidence of folic acid deficiency in pregnancy varies from 2% to 50% in different reported series, and sub-clinical folate deficiency in the mother has been implicated in neural tube defects (spina bifida) in the fetus.** There is powerful evidence that extra folate given very early in pregnancy reduces the number of babies born with spina bifida. In 1996, the U.S. Food and Drug Administration began requiring the addition of folate to cereal grains, so that all who eat bread and cereal would derive added folate. After this policy went into effect, median serum folate levels in the U.S. rose by about 50%, and the incidence of neural tube defects decreased. Concern expressed by some experts that this public health policy decision would result in neurologic disease in some people with undetected cobalamin deficiency has not been borne out.

Folic acid deficiency is common in alcoholics and is probably the result of several factors: decreased intake, decreased absorption, possibly an antagonistic effect of ethanol at the intracellular enzyme level, and probably decreased storage in an injured (cirrhotic) liver. Megaloblastic anemia in alcoholics is almost always caused by folic acid deficiency.

4. Drugs

Some drugs, when taken over an extended period of time, have been associated with an increased incidence of folate deficiency and megaloblastic anemia. Phenytoin, an anticonvulsant taken daily by thousands of people in the Western world, is statistically associated with a lower serum folate level, mild to moderate macrocytosis and, uncommonly, frank megaloblastic anemia. It is not clear whether the tendency to folate deficiency is due to inhibition of the folate deconjugase, or to diminished absorption of the monoglutamate. Treatment of such megaloblastic anemia with modest doses of oral folate is effective.

However, some evidence indicates that rapid correction of the folate deficiency increases the likelihood of seizures in such patients.

Many years ago, severe arteriosclerosis was noted in a child who died of congenital homocysteinuria, a condition manifesting extreme hyperhomocysteinemia. In the last 25 years **moderate/mild elevation of plasma homocysteine (which may accompany either B₁₂ or folate deficiency) has been proven to be a significant, independent risk factor for stroke, myocardial infarct, and peripheral vascular disease.** Such elevations are not rare even in persons who have serum B₁₂ and folate and pyridoxine in the range of normal. Supplemental folate, and to a lesser extent B₁₂ and pyridoxine, will lower the homocysteine level in such patients. **However, current data do not indicate that lowering homocysteine levels improves cardiovascular outcomes.**

5. Treatment of folate deficiency

- Folic acid is available as a generic in oral and parenteral forms.
- Usually given PO; readily absorbed. Folic acid was number 90 on the most-prescribed drug list for 2005.
- 1 mg QD will reverse megaloblastic anemia, restore normal serum folate levels, and replenish body stores in almost all patients.
- Response is rapid and complete and similar to the response of the vitamin B₁₂-deficient patient given parenteral vitamin B₁₂. Anemia should be completely corrected in 1-2 months.
- Essentially nontoxic, but can see CNS effects such as irritability, overactivity, difficulty concentrating, and depression in large, chronic doses.
- For acutely ill patients, begin TX with both vitamin B₁₂ and folic acid IM until full diagnosis is made.

VI. Summary

Both B₁₂ and folic acid contribute to donation of single carbons in interlocking metabolic pathways and megaloblastic anemia due to deficiency of one is identical morphologically to deficiency of the other. Folate has a short half-life in the body and must be replenished frequently. Vitamin B₁₂ deficiency can lead to irreversible neurologic damage.

CHAPTER 4

INTRODUCTION TO HEMOLYSIS; RED CELL PHYSIOLOGY

Key Concepts:

- Definition, causes, clinical consequences, and diagnosis of hemolytic anemia
- Basic principles of red cell metabolism and hemoglobin function.

Learning Objectives:

1. Describe the red cell membrane.
2. Define hemolysis and list the three mechanisms by which it may occur.
3. Be able to distinguish extravascular and intravascular hemolysis using laboratory tests, and to use this information to help determine the cause of hemolysis.
4. Describe the role of the spleen in hemolysis.
5. Be able to use laboratory test results and information from the blood smear and bone marrow biopsy to determine whether hemolysis is present.
6. Describe the complications of hemolysis.
7. Be able to diagnose hereditary spherocytosis using clinical and laboratory information, and to explain its pathophysiology.
8. Describe the biochemical pathways by which the red cell generates energy and maintains membrane integrity.
9. Be able to diagnose glucose 6-phosphatase deficiency based on clinical and laboratory data, and to describe the genetics and pathophysiology of this condition.
10. Describe the effects of pH, CO₂, and 2,3 DPG on the oxygen affinity of hemoglobin, and the physiologic significance of each effect.
11. Describe the effects of carbon monoxide on hemoglobin function and oxygen delivery.

"Comparative hematology suggests that without the red cell membrane, the activity of man could hardly have exceeded the torpid pace of the lugworm."

--Samuel E. Lux. Seminars in Hematology, 16:21, 1979.

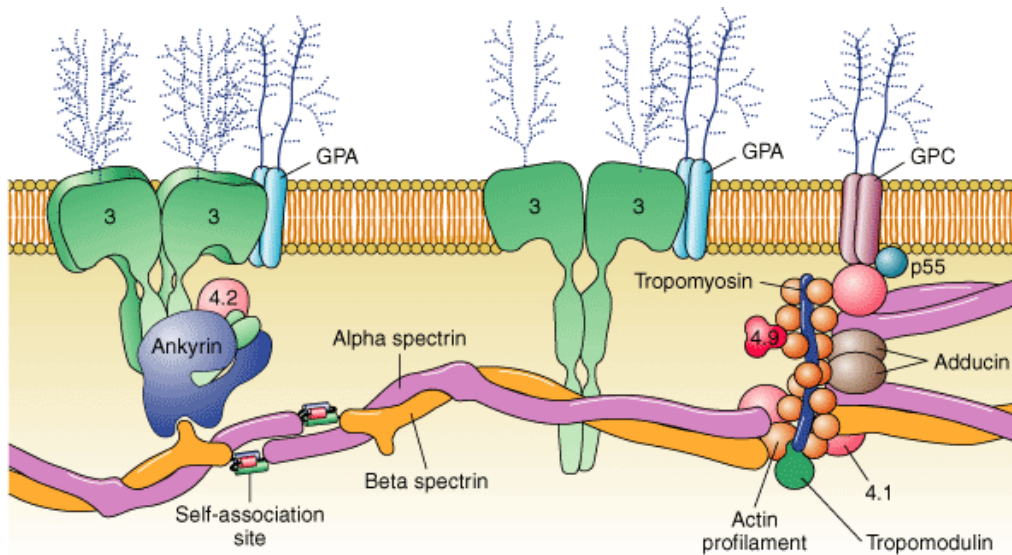
I. The Red Cell Membrane

Many invertebrates have no red cells. Their respiratory pigments are dissolved in the plasma. Among the vertebrates, hemoglobin is confined within a membrane, resulting in the 400% increase in oxygen carrying capacity needed to satisfy the demands of mammalian metabolism.

"Packaging" not only makes high hemoglobin concentration possible, but protects hemoglobin from oxidation and enables red cell 2,3-DPG to modify hemoglobin affinity for oxygen. Having a red cell membrane however, carries the risk of membrane rupture i.e., hemolysis. The next chapters cover the abnormalities of membranes, enzymes, and hemoglobin that can lead to hemolysis.

A. Composition of the Membrane

The red cell membrane is made up of equal quantities of lipids and proteins (Fig. 4.1).



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Fig 4.1 Diagram of a cross-section of the RBC membrane. Spectrin, actin, tropomyosin, adducin, and protein 4.1 form a meshwork that laminates the inner surface of the membrane. In contrast, other proteins such as the glycophorins (GPA and GPC) and protein 3 (anion transport channel) traverse the lipid bilayer. Long polysaccharide chains are covalently attached to these proteins on the outer surface of the cell and also to glycolipid. Ankyrin and protein 4.2 form a bridge between spectrin and a fraction of the anion transport proteins. Protein 4.1 binds to GPC. (From: Lux SE, Palek J, in *Blood: Principles and Practice of Hematology*, RI Handin et al (eds). Philadelphia, Lippincott, 1995.)

B. Lipids

The lipids are organized in an asymmetric bilayer, with the choline phospholipids (phosphatidylcholine, sphingomyelin) concentrated in the outer half of the bilayer and the aminophospholipids (phosphatidyl serine, phosphatidyl ethanolamine) in the inner half. Cholesterol and glycolipids are interspersed among the phospholipids. The membrane cholesterol and phospholipids are partially exchangeable with plasma lipids. The lipid bilayer is in a fluid state permitting **flexibility** of the red cell membrane.

C. Membrane Proteins

The major membrane proteins have been characterized and divided into two groups: the integral proteins and the structural proteins.

1. Integral Proteins

The integral proteins traverse the lipid bilayer or are attached to it by a glycolipid anchor.

These include:

- receptor and antigen-bearing proteins such as glycophorin A
- transport proteins such as sodium-potassium ATPase
- the anion transport protein, which facilitates chloride and bicarbonate movement

- “channel” proteins that facilitate diffusion of glucose into the cell
- glycolipid anchored proteins such as membrane inhibitor of reactive lysis (MIRL or CD59), which protect the red cell from lysis by complement.

2. Structural Proteins (cytoskeletal proteins)

The structural proteins are confined to the inner surface of the membrane and consist primarily of spectrin, actin, and ankyrin, as well as some enzymes. The spectrin-actin network supports the lipid bilayer and stabilizes the position of the membrane-spanning proteins. This network thus assumes a dominant role in maintaining membrane shape and flexibility.

D. Accessory Membrane Functions

To maintain its osmotic equilibrium, the red cell must actively pump out sodium and water. Extrusion of Na^+ and inward transport of K^+ are accomplished by the membrane enzyme $\text{Na}^+ - \text{K}^+$ ATPase. The erythrocyte also maintains a very low intracellular concentration of calcium, actively extruding it via the membrane $\text{Ca}^{++} - \text{Mg}^{++}$ ATP dependent calcium pump.

II. Hemolysis: General Concepts

A. Definition

The normal human red cell emerges from the marrow and circulates in the peripheral blood for approximately 120 days (100 to 130 days). At the end of its life span, it is removed from the circulation by macrophages of the liver and spleen. Hemolysis is defined as an erythrocyte survival time less than 100 days.

B. Mechanisms of Hemolysis

There are three basic pathophysiologic mechanisms of hemolysis:

1. **loss of red cell deformability.**
2. **antibody attachment to red cell surface antigens.**
3. **creation of holes in the red cell membrane.**

When red cells from the circulation are removed prematurely by macrophages of the liver and spleen, as in the first and second mechanisms, hemolysis is "**extravascular.**" When red cells rupture within the blood stream, as in the third mechanism, the process is known as "**intravascular**" hemolysis.

1. Loss of Red Cell Deformability

During its 120-day life span, the erythrocyte travels up to three hundred miles, traversing high-pressure arterial systems and squeezing through capillaries and sinusoidal walls that are only two to three microns in diameter. In order to negotiate its seven- to eight-micron diameter through spaces one third its size, **the red cell must be extremely flexible.** Flexibility plays a critical role in red cell survival, and in its ability to deliver oxygen. (Fig 4.2)

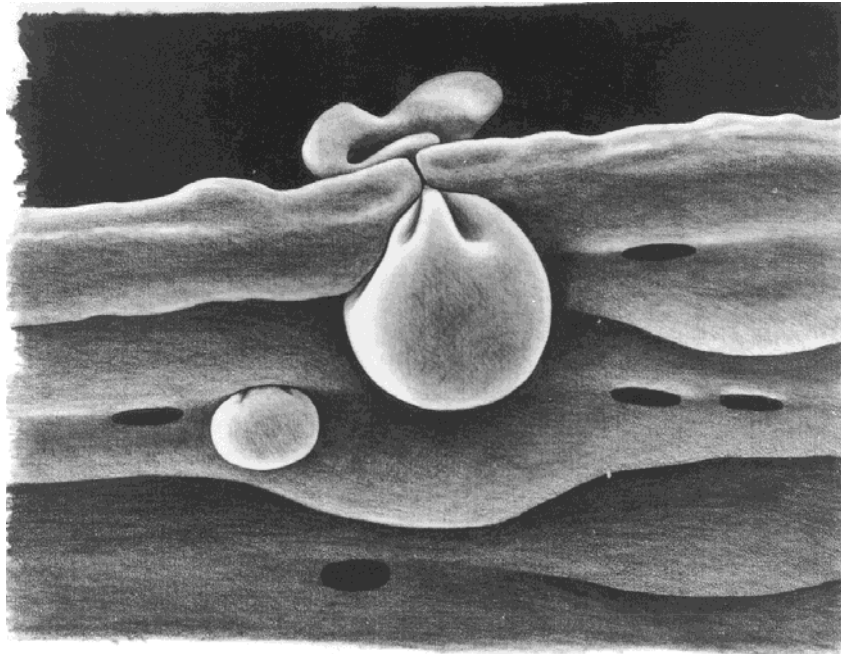


Figure 4.2. Red cells squeezing through a rat spleen sinusoidal slit. Redrawn from a scanning electron micrograph. (From Mohandas N et al.: Red blood cell deformability and hemolytic anemias, *Sem. Hematol.* 16: 1

Flexibility or deformability of the red cell is dependent on four variables:

- **the viscoelastic properties of the membrane**, which in turn are dependent mainly on the integrity of the spectrin-actin cytoskeleton;
- **the surface-to-volume (S:V) ratio of the cell** (the more spherical the cell, i.e., the lower its S:V ratio, the less deformable it is);
- **the mean corpuscular hemoglobin concentration of the erythrocyte** (an increase in intracellular hemoglobin concentration decreases deformability);
- **the physical state of the hemoglobin within the cell.** (Polymerization or precipitation of intracellular hemoglobin, as seen in sickle cell anemia, G-6-PD deficiency, or thalassemia, impairs red cell flexibility.)

Any process that decreases a red cell's deformability will impede its passage through the tiny slits in the splenic sinusoidal walls. Macrophages surrounding the sinusoids will phagocytize the trapped cell, causing extravascular hemolysis.

2. Phagocytosis of Antibody and Complement-Coated Red Cells.

IgG and IgM antibodies that attach to red cell membrane antigens cause phagocytosis of red cells by macrophages of the liver and spleen. See chapter 7 for more detail.

3. Disruption of Membrane Integrity: Intravascular Hemolysis

Intravascular hemolysis occurs when holes appear in the red cell membrane. Membrane leaks are made when:

- hemolytic complement (C5-C9) is fixed to the membrane, as in **ABO mismatched transfusion reactions**;
- membrane sulfhydryl groups are oxidized, as in **severe G-6-PD deficiency**;
- a parasite invades and subsequently emerges from a red cell (**malaria**);
- the erythrocyte is mechanically cut or abraded within the vascular system by fibrin (**microangiopathic hemolytic anemia** or abnormal heart valves).

Big holes (larger than 32.5 angstroms) allow hemoglobin to leak out of the red cell. Smaller holes allow water to move in to equalize the osmotic gradient produced by the high concentration of intracellular hemoglobin. In either case, the red cell bursts, and hemoglobin and red cell membrane fragments are released directly within the vascular space, i.e., "intravascular hemolysis."

C. Normal Pathway of Red Cell Breakdown

Two possible mechanisms explain how macrophages recognize and destroy aged normal red cells: the development of spherocytosis, and the attachment of antibody.

1. The activities of various intracellular enzymes decrease as a red cell ages. As a consequence, the concentration of ATP decreases, depriving the calcium pump of its energy source, and intracellular calcium levels rise. The result is fragmentation and loss of bits of the cell membrane causing spherocytosis. This in turn causes decreased deformability and ultimate entrapment by spleen and liver macrophages.
2. With normal red cell aging there are alterations of membrane sialoglycoproteins. An IgG autoantibody present in normal human serum attaches to these altered membrane proteins. When a sufficient level of antibody coating is reached, macrophages recognize the red cell as foreign, and phagocytize it.

When the red cell is engulfed by a macrophage, the red cell membrane is digested, and hemoglobin is broken into its three component parts--globin, iron, and protoporphyrin (Fig 4.3). The globin chains are degraded, and their amino acids recycled in the body's amino acid pool.

The iron is zealously conserved. Serum transferrin carries it from the macrophage back to the marrow for hemoglobin synthesis, or to macrophage storage sites in the liver, spleen, and marrow. **The protoporphyrin moiety is converted to bilirubin**, which diffuses out of the macrophage and complexes with serum albumin, now called "**indirect**" or "**unconjugated**" **bilirubin**.

The bilirubin-albumin complex is transported to the liver hepatocyte, where the bilirubin is conjugated with glucuronide (now called "**direct**" or "**conjugated**" **bilirubin**) by the enzyme glucuronyl transferase. The water-soluble conjugated bilirubin is excreted via the bile into the gastrointestinal tract. During the conversion of one mole of protoporphyrin to bilirubin, one mole of carbon monoxide is released and expired in the lungs. This is the principal reaction producing carbon monoxide in the body.

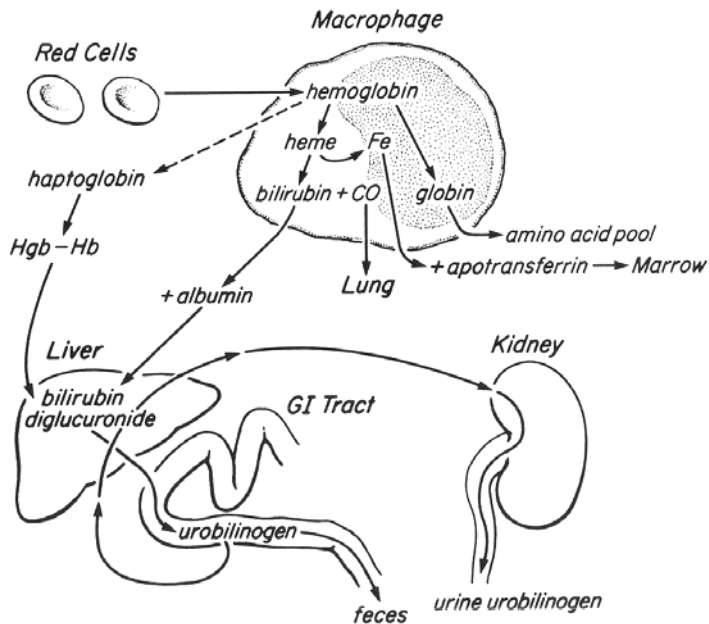


Figure 4.3. Extravascular hemolysis. Almost all hemoglobin is degraded intracellularly and its products recycled. Traces of hemoglobin are bound to haptoglobin. The bilirubin pathway uses both bowel and kidney for excretion of the residual porphyrins.

D. Extravascular Versus Intravascular Hemolysis

1. Extravascular hemolysis

In extravascular hemolysis, destroyed red cells are processed by the spleen and liver in the same manner as normal senescent red cells. However, because of the rapid breakdown of red cells during hemolysis, the capacity of the liver to conjugate the increased burden of bilirubin may be exceeded, and **serum levels of unconjugated (indirect) bilirubin may rise.**

2. Intravascular hemolysis

During intravascular hemolysis, hemoglobin is degraded by different pathways (Fig.4.4). Normal plasma contains **haptoglobin**, a protein that can bind 100 to 140 mg/dl of free hemoglobin or about 1% of the hemoglobin in red cells. Plasma hemoglobin binds to haptoglobin to form a complex that is rapidly cleared by the hepatocytes. Serum haptoglobin levels therefore will be very low or absent in intravascular hemolysis. Even in brisk extravascular hemolysis, enough hemoglobin leaks out of the macrophages to bind with and deplete haptoglobin. Therefore, **a low serum haptoglobin concentration is a good indicator of hemolysis.**

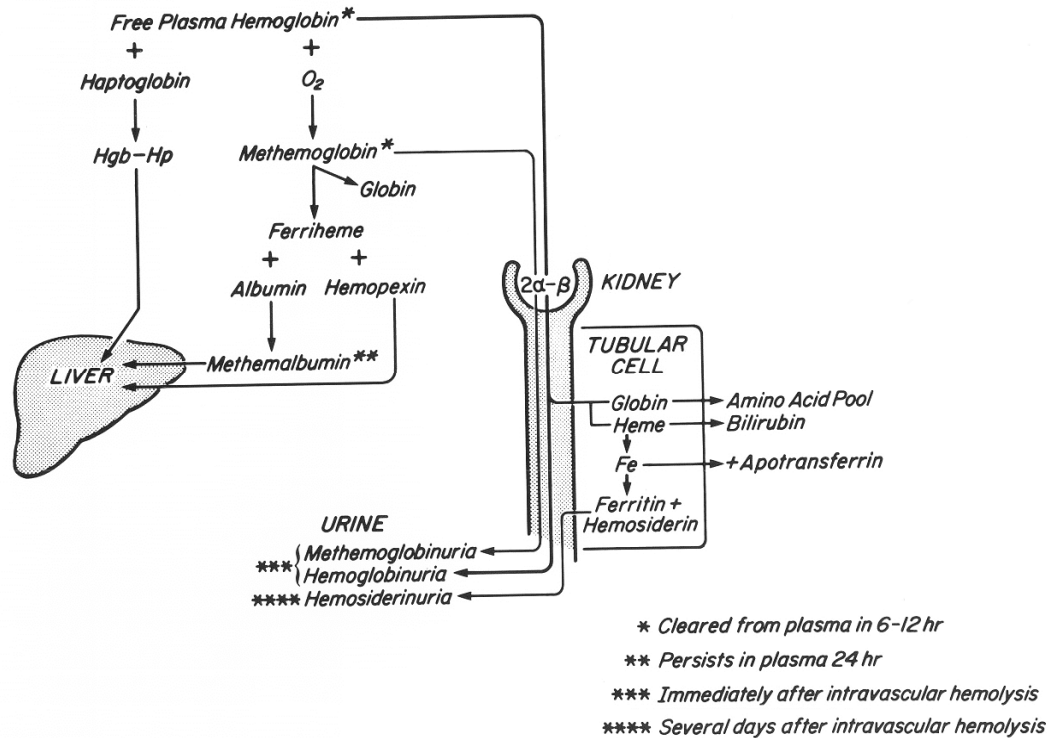


Figure 4.4. Intravascular hemolysis. The time of appearance and clearance of hemoglobin and its products is indicated by asterisks.

After saturating haptoglobin, some of the remaining free hemoglobin is oxidized to methemoglobin, most of which is excreted in the urine.

The remaining free intravascular hemoglobin is filtered in the urine. A portion of this filtered hemoglobin is reabsorbed by the renal tubular cells, where globin is degraded to amino acids, and protoporphyrin is converted to bilirubin. Most of the iron remains in the tubular cell in the form of ferritin or hemosiderin. When the tubular cell exfoliates into the urine, the iron is lost with it and may be seen in urine sediments stained for iron with Prussian blue. Thus **hemosiderinuria is a strong indicator of intravascular hemolysis**. When intravascular hemoglobin release is brisk, the hemoglobin filtered by the glomerulus cannot all be reabsorbed by the tubular epithelium. Free hemoglobin appears immediately in the urine, producing a red-brown color. **The most specific indicators of intravascular hemolysis are the presence of free hemoglobin in the plasma and/or urine.**

In conclusion, whereas in chronic extravascular hemolysis iron is tenaciously conserved, in chronic intravascular hemolysis, large amounts of iron are lost in the urine as free hemoglobin, methemoglobin, and hemosiderin, and the patient may become iron-deficient. Other complications of hemolysis are covered later in this chapter.

E. Role of the Spleen in Hemolysis

The spleen is an organ with both lymphoid and macrophage functions. Because of its unique "open" circulation, it acts as an efficient filter to remove even minimally defective red cells from the circulation. The spleen has a capsule traversed by arterial vessels that branch repeatedly, finally reaching the splenic cords (Fig. 4.5). These cords are bands of macrophage-rich tissue lying between the splenic sinuses.

Although the majority of the blood flowing through the spleen travels rapidly, as if in enclosed vessels, a smaller portion travels slowly. The slower-moving red cells are deposited in the splenic cords, where they meander across and then insinuate themselves through the one-to three-micron slits in the sinus wall. The sinuses anastomose freely and then empty into the splenic veins. If an erythrocyte is stiff and cannot pass through the sinus wall slits, the cord macrophage will phagocytise it.

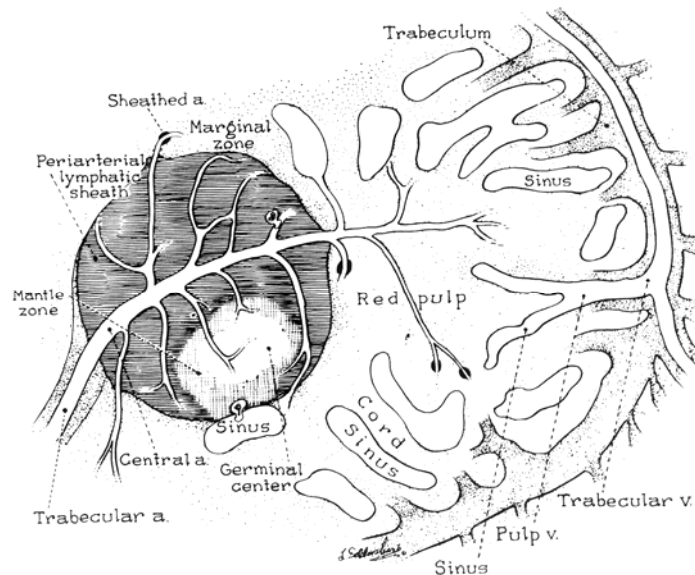


Figure 4.5. Diagram of the organization of blood vessels in the spleen. (From Weiss L: The Spleen, in Greep, RO, Weiss, L (eds): *Histology*, New York, McGraw-Hill, p. 445.)

The metabolic environment of the spleen "conditions" slightly abnormal red cells, making them even more rigid. As blood passes the white pulp, plasma is skimmed off so that blood entering the cords has a high red cell concentration, sluggish flow, and a decreased supply of substrates. **The splenic cord is hypoxic, acidotic, and hypoglycemic. A red cell caught in this environment is less able to generate ATP, and may become spherocytic and be phagocytized. Red cells with relatively minor abnormalities, as in hereditary spherocytosis, can successfully pass through the circulation in every part of the body except the spleen (the body's fine filter). Splenectomy in such patients will eliminate the hemolysis.** More seriously deformed red cells, such as sickled cells, will also be removed by the macrophages of the liver (the body's coarse filter) and splenectomy will be ineffective.

F. Role of Antibody and Complement in Hemolysis

Immune hemolysis is discussed in detail in Chapter 7. In this chapter, only a few preliminary points will be made. Antibodies that attach to red cell surface antigens may be of the immunoglobulin classes IgG or IgM. The antibody may not fix complement at all, fix complement only through C3b, or bind to completion through C9. The class of antibody, the concentration of antibody, and whether or not the antibody fixes complement will determine if the red cell is removed by the liver and / or by the spleen.

Any antibody that fixes complement through C9 will cause intravascular hemolysis (Fig.4.6). Otherwise, antibody-mediated hemolysis is mainly extravascular, as described in Chapter 7.

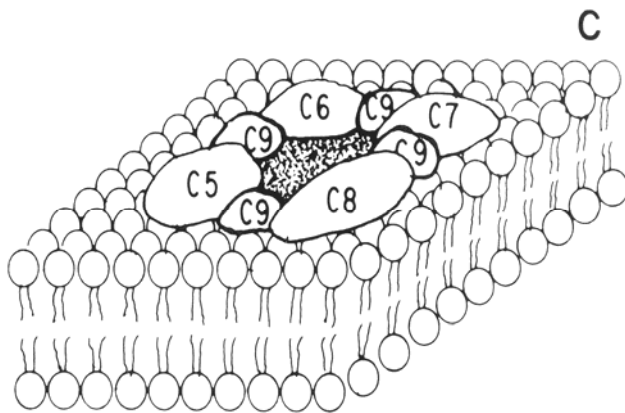


Figure 4.6. Diagram of electron micrograph of holes in the red cell membrane created by complement. (From Rosse, W: Interactions of complement with the red cell membrane. *Sem. Hematol* 16:133.

G. Marrow Response to Hemolysis

Erythroid hyperplasia is seen in the marrow within two days of an acute hemolytic episode. The reticulocyte count begins to rise in three to four days, but a new steady state is achieved only after two to three months of chronic hemolysis. The normal marrow is capable of increasing its daily output of red cells at least fivefold in response to chronic hemolysis. Thus, a red cell life span as short as 20 days may be associated with a normal hemoglobin. This is called "**compensated hemolysis.**" Further decreases in life span are accompanied by anemia. Under intense stimulation, the marrow becomes very hypercellular, erythroid elements replace marrow fat, and active marrow expands in the axial skeleton, long bones, and skull.

In children with severe hereditary hemolytic anemias, hematopoiesis may resume in organs such as the liver and spleen that produced red cells in the embryo. Tumor-like masses of erythropoietic cells also may develop along the vertebrae, representing extensions of marrow through the thin vertebral bony cortex. Production of blood cells outside the marrow cavity is known as **extramedullary hematopoiesis.**

When hemolysis is extravascular, iron released from the broken-down red cells is efficiently shuttled back to the erythroid marrow for the manufacture of new hemoglobin. The expansion of marrow mass and the efficient recycling of iron account for the ability of the marrow to increase red cell production to as much as 5-8x normal.

III. Complications of Hemolysis

A. Anemia

If the red cell life span is less than 20 days, anemia results. (If there is any sort of marrow impairment, anemia will occur with lesser degrees of hemolysis.) Symptoms of weakness, fatigue, and shortness of breath may ensue.

B. Aplastic Crisis

In association with **Parvovirus B19** infections, anyone may have a temporary, selective failure of red cell production. This is a **self-limited red cell aplasia** lasting about a week, associated with a flu-like illness. A normal person replaces 1/120 of the red cells daily. If a normal marrow shuts down for seven days, there will be a trivial fall in hematocrit of 7/120, from 45% to approximately 42.5%. On the other hand, a patient whose red cell survival is only 10 days destroys and replaces 1/10 of the cells daily. If erythrocyte production ceases for a week while destruction continues at the previous rate, approximately 7/10 of the red cells will be destroyed and the hematocrit will fall precipitously. Such an aplastic crisis may occur in any chronic hemolytic anemia, and is recognized by the disappearance of reticulocytes from the blood and normoblasts from the marrow. Transfusions are required until the patient's own red cell production resumes.

C. Folic Acid Deficiency

Folic acid requirements are increased in chronic hemolysis and may exceed the amounts supplied by a normal diet. The occurrence of folate deficiency is marked by worsening anemia, decreasing reticulocyte counts, hypersegmented neutrophils and macrocytes on peripheral smear, and megaloblastic changes in the marrow. **All patients with chronic hemolysis should be given a daily folic acid supplement.**

D. Skeletal Abnormalities

When the marrow space is massively expanded, it can deform surrounding bones. This is especially notable in the skulls of growing children, producing the "hair-on-end" appearance of the skull X-ray. Forehead bossing, broad cheek bones, and protruding maxillae produce a characteristic "hemolytic" or "chipmunk" appearance in children with severe hereditary hemolytic anemia.

E. Kernicterus and Gallstones

The enormous increase in bilirubin production due to rapid heme degradation in hemolysis may have untoward effects. **In the newborn, hemolysis (Rh incompatibility, hereditary enzyme defects) is particularly hazardous.** Hepatic glucuronyl transferase activity is low, and unconjugated bilirubin accumulates in the plasma and gains access to the brain. Basal ganglia are vulnerable because they are incompletely myelinated at birth. Serious neurologic sequelae including seizures, choreoathetosis, and mental retardation may ensue. Kernicterus may be prevented by performing exchange transfusions when serum indirect bilirubin levels approach 20 mg/dl, or by exposing the infant to ultraviolet light.

Gallstones are a common finding in patients with chronic hemolytic disorders. These are black, bilirubin-containing stones, and may occur even in children and young adults with hereditary hemolytic disorders. (Gallstones in older adults are composed of cholesterol and do not suggest hemolysis.)

F. Iron Overload

Patients with extravascular hemolysis retain all the iron liberated during red cell degradation. Furthermore, they absorb more dietary iron than normal, especially when ineffective erythropoiesis (as in thalassemia) is also present. If the patient also requires red cell transfusions to grow and function, body iron stores rapidly increase. The excess iron is initially deposited in macrophages, but with accumulations of 25 to 50 grams of iron in adults (100 to 250 transfusions), deposition occurs in parenchymal cells of the heart, pancreas, liver, and endocrine glands, leading to fibrosis of these organs. Transfusion hemosiderosis is an important cause of death in thalassemia major (see Chapter 5). Most other chronic hemolytic anemias are less severe and require transfusion only during aplastic crises or surgery, and hemosiderosis only rarely develops

G. Complications Unique to Intravascular Hemolysis

During acute intravascular hemolysis, large amounts of free hemoglobin and red cell membrane fragments are liberated in the blood. Free hemoglobin itself probably is not very harmful, but membrane lipids such as phosphatidyl serine and phosphatidyl ethanolamine can activate clotting factors and initiate disseminated intravascular coagulation (DIC). If an antigen-antibody reaction causes the intravascular hemolytic episode (ABO-incompatible blood transfusion), complement activation occurs, with the liberation of C3a and C5a. These small polypeptides act directly on vascular smooth muscle and also release vasoactive substances from mast cells, thus producing renal vasoconstriction and shock. **Acute intravascular hemolytic reactions therefore, may be associated with DIC, acute renal failure, shock, and death.** (see chapter 6 on hemolytic transfusion reactions, and chapters 9 and 10 on DIC)

IV. Diagnosis of Hemolysis

A. Peripheral Smear

The peripheral smear is very valuable for the detection and evaluation of hemolysis. Large numbers of polychromatophilic macrocytes (shift cells), spherocytes, elliptocytes, spur cells (see below), target cells, sickled cells, or schistocytes (mechanically damaged cells and cell fragments – see below) may immediately suggest the correct diagnosis.

B. Reticulocytosis

A persistently high reticulocyte count is the best clinical test of hemolysis. Exceptions to this rule include aplastic crisis or folate deficiency, or when the hemolytic episode has occurred so recently that the marrow has not had time to increase production.

C. Measurement of the Products of Red Cell Destruction

Increased indirect (unconjugated) bilirubin and low or absent serum haptoglobin levels are found in most patients with brisk hemolysis. Elevated serum levels of the enzyme lactic dehydrogenase (LDH) also are common. This enzyme, present in high concentration in normal red cells, is released when the red cell is destroyed.

Transiently, hemoglobin, methemoglobin, and methemalbumin will be found in the plasma after intravascular hemolysis. Methemoglobinuria and hemoglobinuria may be seen immediately after intravascular hemolysis, and hemosiderinuria will follow the episode in a few days and may still be detected two to four weeks later.

The peripheral smear and reticulocyte count are the best screening tests to detect hemolysis. The indirect bilirubin, haptoglobin, and LDH are also useful. More specific tests, such as osmotic fragility, hemoglobin electrophoresis, and the Coombs (antiglobulin) test, will be described subsequently.

V. Classification of Hemolytic Disorders

Several classifications have been proposed, none of which is completely consistent. Disorders have been grouped according to whether they are hereditary or acquired, whether hemolysis is intravascular or extravascular, and whether the abnormality resides within the cell itself (intracorporeal) or in the environment of the cell (extracorporeal).

See Table 4.1 for a list of hemolytic diseases. In this table, the entities that are emphasized in this course are in bold type.

Table 4.1

CLASSIFICATION OF HEMOLYTIC DISORDERS

(Adapted from Harris, Kellermeyer: *The Red Cell*, rev. ed.)

- I. Intracorpuseular Abnormalities
 - A. Membrane
 - 1. Hereditary: **hereditary spherocytosis**, hereditary elliptocytosis, hereditary stomatocytosis.
 - 2. Acquired: paroxysmal nocturnal hemoglobinuria (PNH).
 - B. Hereditary defects of globin synthesis
 - 1. **Hemoglobinopathies--SS, SC, CC**, unstable hemoglobins, etc.
 - 2. **Thalassemias.***
 - C. Hereditary enzyme defects
 - 1. Glycolytic pathway:
 - pyruvate kinase, hexokinase
 - triose isomerase
 - glucose phosphate isomerase.
 - 2. Pentose phosphate pathway:
 - **G-6-PD deficiency.**
- II. Extracorpuseular Abnormalities
 - A. Associated with antibodies (acquired)
 - 1. Isoantibodies:
 - **erythroblastosis fetalis**
 - **transfusion reactions.**
 - 2. Autoantibodies: (Autoimmune Hemolytic Anemia)
 - **Warm-reacting** (idiopathic, or associated with lymphoma, chronic lymphocytic leukemia, systemic lupus erythematosus)
 - **Cold agglutinin disease** (idiopathic, or associated with mycoplasma pneumonia, infectious mononucleosis, lymphoma)
 - Cold hemolysin (paroxysmal cold hemoglobinuria)
 - **Drug-induced antibodies**
 - B. Unassociated with antibodies
 - 1. Disorders of serum lipids:
 - Hereditary; abeta lipoproteinemia
 - Acquired: spur cell anemia (liver disease)

2. Traumatic (microangiopathic) hemolysis (acquired):
 - **Disseminated intravascular hemolysis**
 - **Thrombotic thrombocytopenic purpura (TTP)**
 - **Hemolytic uremic syndrome (HUS)**
 - Pre-eclampsia
 - Malignant hypertension
 - Cardiac valvular abnormalities or prostheses
 - "March" hemoglobinuria
3. Infectious agents (acquired):
 - Bacterial toxins (*clostridium Welchii*, bacteroides)
 - Parasites (malaria, bartonella)
4. Chemical, physical toxins (acquired):
 - Heavy metals
 - Arsine
 - Naphthalene
 - Intravenous distilled water
 - Burns

III. Interaction of Intracorporeal and Extracorporeal Defects (hereditary intracorporeal defects, requiring an environmental exposure to induce hemolysis)

- A. **G-6-PD deficiency**
- B. Favism
- C. Certain unstable hemoglobins

*In thalassemia, the main mechanism of anemia is ineffective erythropoiesis, with hemolysis a major secondary mechanism.

VI. Disorders of the Red Cell Membrane

A. Abnormalities of Structural Proteins

As described above, the red cell membrane consists of an asymmetric lipid bilayer that is traversed by a number of different transmembrane receptor and antigen bearing proteins. On the inner aspect of the lipid bilayer is the spectrin-actin cytoskeleton, which controls the shape and deformability of the red cell. Since membrane skeletal proteins are linked to some of the transmembrane receptor proteins, the skeletal proteins may also be responsible for transmitting signals from growth factors and hormones into the cytoplasm of the red cell.

The major proteins in the cytoskeleton are spectrin, actin, protein 4.1, and ankyrin. (Fig. 4.1) These proteins support the lipid bilayer and stabilize the positions of the membrane-spanning proteins.

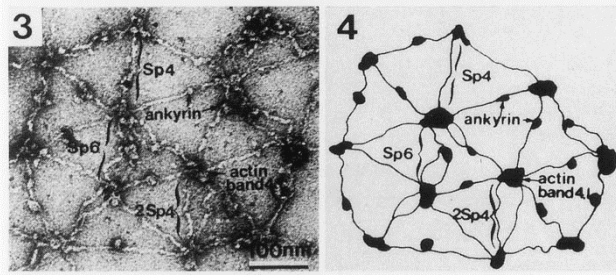


Figure 4.7. Ultrastructure of the red cell membrane: The knitted lattice is made up of pentagons, hexagons, and heptagons with spectrin tetramers (Sp4), hexamers, (Sp6) and double tetramers. (2Sp4) linked to actin-band 4.1 junctions. Ankyrin globules are seen in the middle of the spectrin threads. From Palek and Lambert, *Genetics of the red cell membrane skeleton. Sem Hematol* 27, 290.

From the complex nature of the spectrin-actin-4.1-ankyrin-transmembrane protein interactions (Fig. 4.1, 4.7), it is easy to see opportunity for error. In fact, a variety of such defects do occur causing the clinical disorders **hereditary spherocytosis** and **hereditary elliptocytosis**.

Hereditary Spherocytosis (HS)

Hereditary spherocytosis is a spectrum of hemolytic disorders produced by several genetic abnormalities. The common form of hereditary spherocytosis is an **autosomal dominant** hemolytic disease that affects approximately one in five thousand in the U.S. population. Although found in all races, it is most prevalent among Northern Europeans. It is frequently associated with defects of chromosome 8 at the site of the ankyrin gene. It is believed that **abnormalities or deficiencies of ankyrin lead to a deficiency of spectrin. Spectrin deficiency causes loss of membrane with formation of small spherical red cells termed “microspherocytes” or spherocytes** (Fig. 4.8).

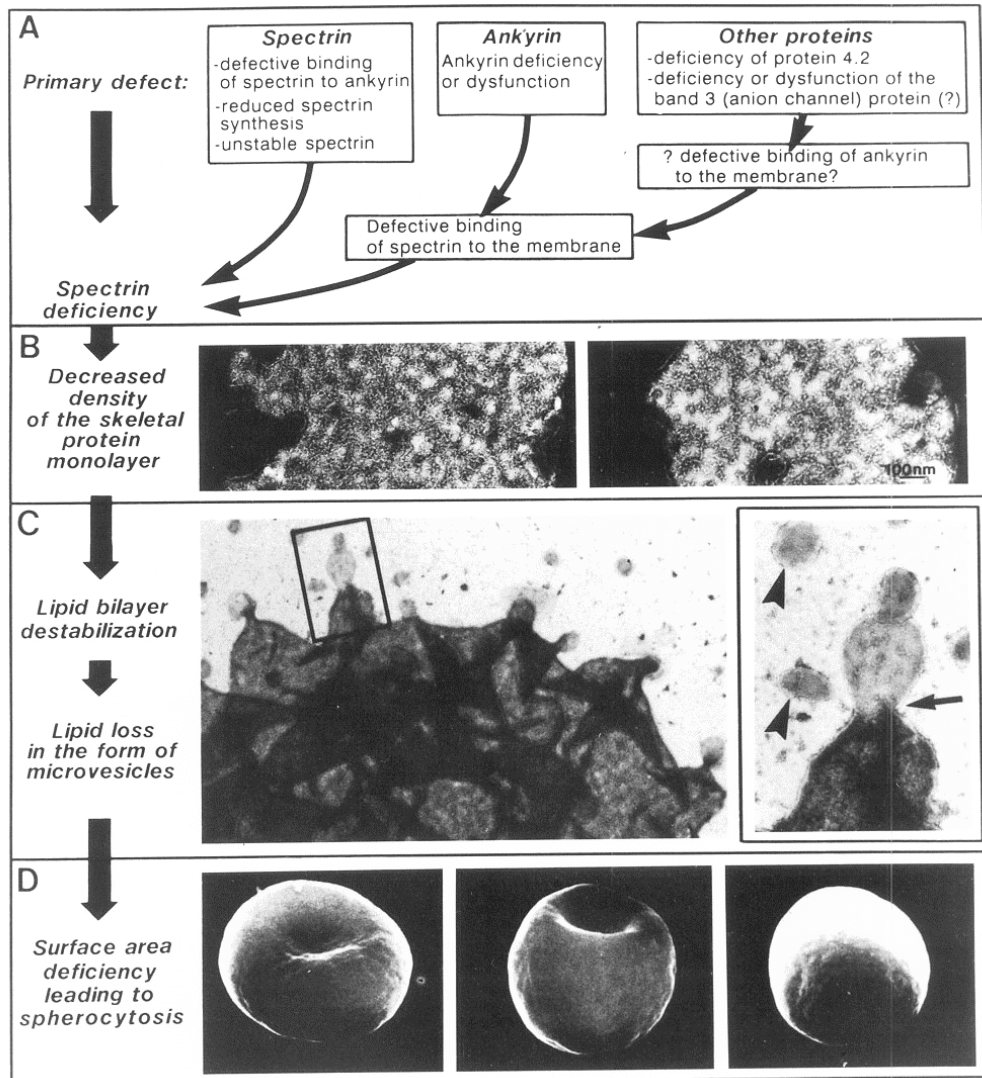


Figure 4.8. Pathophysiology of the red cell defect in hereditary spherocytosis. B shows the reduced density of the H.S. cytoskeleton on the right compared with the normal on the left. C shows the lipid layer separating from the abnormal skeleton (arrow) with micro-vesicles free in the medium (arrow-heads). From Palek & Lambert. Genetics of the red cell membrane skeleton. *Sem Hematol* 27, 290.

There is a direct relationship between the degree of spectrin deficiency and the severity of the disease. **The spherocyte has a high intracellular hemoglobin concentration (MCHC) as well as a low surface-to-volume ratio, two features that make the cell poorly deformable.** In addition, the HS cell membrane is excessively permeable to sodium. To overcome this increased permeability, the cell requires extra glucose to maintain its $\text{Na}^+\text{-K}^+$ pump activity. Because of the lack of flexibility of the spherocyte and its dependence on accelerated glycolysis to compensate for the membrane sodium leak, **the spleen presents the main threat to its survival.**

When the spherocyte enters the spleen, it is detained in the splenic cord, where glucose levels are very low and intracellular ATP falls rapidly. $\text{Na}^+ - \text{K}^+$ pump activity cannot be sustained, and sodium and water enter the cell, causing further spherizing. **After several passes through the spleen, the cell becomes “hyperspheroidal.” It cannot negotiate the sinusoidal slits, and it is phagocytized by splenic macrophages.**

1. Clinical Picture

The clinical spectrum is variable. Patients may have a mild anemia, splenomegaly, and jaundice, but many are asymptomatic and undiagnosed, even in old age. A newborn may require exchange transfusion to prevent kernicterus. Bilirubin gallstones occur in a large proportion of patients, even in childhood, and cholecystitis may be the first clinical evidence of the disease. As in other hemolytic disorders, aplastic crises and folic acid deficiency may occur.

2. Laboratory Findings

The peripheral smear provides an important clue to diagnosis. Spherocytes--dark round cells without central pallor--comprise at least 20% of the cells. However, in some proven cases, spherocytes are not conspicuous and other tests must be used. As in other causes of hemolysis, there is **reticulocytosis**, and this is a simple test for screening family members. Elevated serum indirect bilirubin is found in many patients. Typical abnormalities should be found in one parent and about half of siblings.



spherocytes

3. Osmotic Fragility Test

The osmotic fragility test is helpful in the diagnosis of hereditary spherocytosis. It indirectly measures the surface-to-volume ratio of the cells by determining how much water the cells can accommodate before they rupture. A spherocyte, with its low surface-to-volume ratio, can accumulate less water than a biconcave disc. Red cells from the patient and a normal control are suspended in varying concentrations of saline, from 0.85% (isotonic saline) to 0 (distilled water). Hereditary spherocytosis cells usually begin to lyse at around 0.65%, whereas normal cells do not hemolyze until around a 0.5% concentration of saline is reached (see Fig. 4.9).

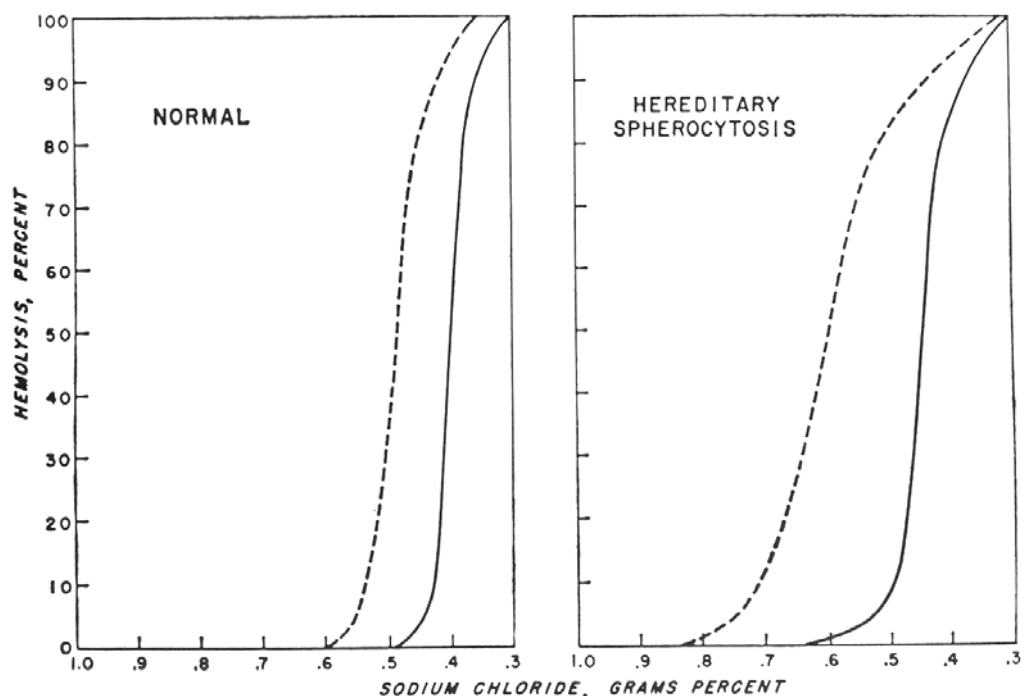


Figure 4.9. Osmotic fragility curves of normal red cells (left) and red cells of hereditary spherocytosis (right). Curves depict the percentage of red cells hemolyzed after incubation in various concentrations of NaCl. Solid lines depict results with freshly drawn red cells. Dashed lines depict results after 24 hours of incubation at 37 °C, which depletes the cells of glucose, causing a decrease in Na⁺-K⁺ pump activity. Isotonic NaCl is 0.85%.

4. Treatment

Because spherocytes are trapped only by the spleen, splenectomy eradicates the hemolysis and anemia resolves. The basic membrane defect persists however, and microspherocytes are still present in the blood. As the spleen has a critical role in phagocytosis of encapsulated bacteria, especially in children, splenectomy should be delayed at least until age 5. After this, the small risk of overwhelming *Streptococcus pneumoniae* sepsis can be reduced by the administration of pneumococcal vaccine, given several weeks prior to surgery. Many patients with hereditary spherocytosis have mild or no anemia (compensated hemolysis), few or no symptoms, and do not require splenectomy.

FYI

Hereditary elliptocytosis (ovalocytosis)

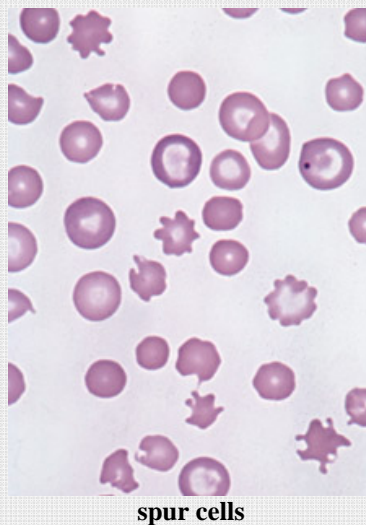
Similar to hereditary spherocytosis, it is also inherited as an autosomal dominant. Again, its genetics and clinical features are quite heterogeneous, but the most common variety is due to a defect in spectrin-spectrin self-association. The abnormal cells are cigar-shaped and have increased osmotic fragility. Although more common than HS (one per 2,500 U.S. population), only 15% of patients have overt hemolysis. Hemolysis, where present, is eliminated by splenectomy.

B. Abnormalities of Membrane Lipids

FYI

Spur cell anemia

As previously noted, red cell membrane lipids are exchangeable with plasma lipids. Spur cell anemia is an acquired disorder of plasma lipoprotein metabolism seen in patients with severe liver disease. In these patients, plasma low-density lipoproteins have a high cholesterol-to-phospholipid ratio. The erythrocyte membrane accumulates cholesterol and develops a bizarre spiculated appearance (acanthocyte). Brisk hemolysis occurs, primarily in the spleen.



VII. Hemolysis Due to Fragmentation of the Membrane: The Traumatic Hemolytic Anemias

Hemolysis due to intravascular trauma to red cells may occur in the heart, the large vessels, or the microvasculature. Fibrin is believed to be the cause of the red cell injury. Abnormal or prosthetic heart valves or arterial grafts accumulate fibrin, which may cause the red cells to shear as they pass these shaggy, roughened surfaces. Microvascular endothelial damage, as seen in malignant hypertension or vasculitis, may cause fibrin strands to be deposited across small arterioles and capillaries. As the erythrocyte travels through these small vessels it is sliced by the fibrin strand like a pancake hitting a wire (Fig. 4.10). The membrane of the fragment may reseal, producing the schistocyte. Some of the fragments do not reseal, and hemoglobin is released intravascularly.

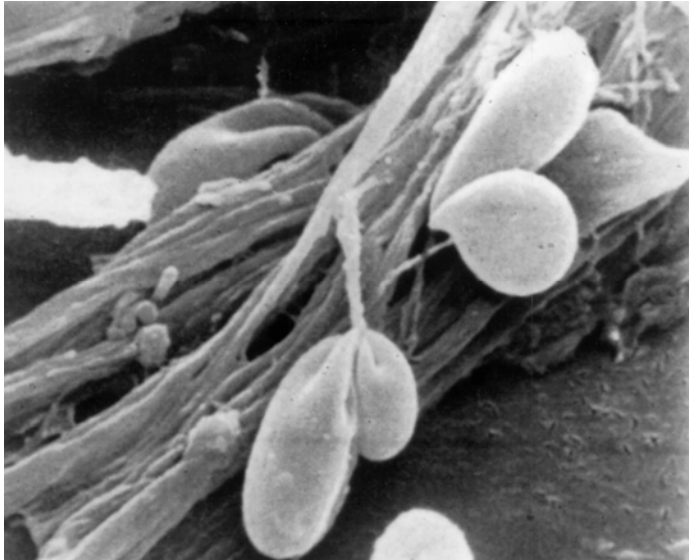
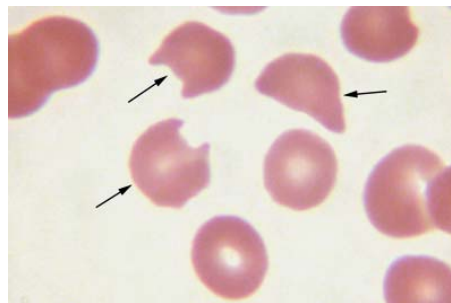


Figure 4.10. An erythrocyte being cut by a fibrin strand in an *in vitro* model of microangiopathic hemolytic anemia. (From Bull BS, Kuhn IN: The production of schistocytes by fibrin strands: a scanning electron microscope study. *Blood* 35, 104).

The blood smear is diagnostic. The hallmark of the traumatic hemolytic anemias is the appearance of schistocytes, or red cell fragments in the peripheral blood. Helmet shapes, triangles, spherocytes, and other small pieces may be seen. **Hemolysis is primarily intravascular with consequent hemosiderinuria and negative iron balance.**



schistocytes

A. Traumatic Cardiac Hemolytic Anemia

Most patients with prosthetic aortic and mitral valves have chronic, mild, well-compensated intravascular hemolysis. However, severe hemolysis may occur when an edge of an aortic prosthesis separates from the root of the aorta. Blood passing through the roughened slit at systolic velocity and pressure may be badly fragmented.

B. Microangiopathic Hemolytic Anemias

In this group of disorders, fibrin strands or platelet clots are strung across the microvasculature, or there is severe endothelial damage in the small vessels, causing red cells to fragment as they pass. One life threatening condition in which microangiopathic hemolytic anemia occurs is thrombotic thrombocytopenic purpura (TTP). In TTP, platelet thrombi occlude the microvasculature. These thrombi are believed to cause the clinical pentad of:

- renal failure
- fluctuating neurologic signs (seizures, paresthesias, coma)
- fever
- thrombocytopenia
- microangiopathic hemolytic anemia.

A similar disorder of small children is called the hemolytic uremic syndrome (HUS). It differs from TTP in that neurologic disease is rare, it often follows a viral upper respiratory infection or a *Shigella* or *E. coli* gastroenteritis, and it is only rarely fatal.

DIC, vasculitis, malignant hypertension, disseminated carcinoma, and several complications of pregnancy (pre-eclampsia, abruptio placenta) also may produce microangiopathic hemolytic anemia. See Chapters 9 and 10 for more detail.

FYI

March Hemoglobinuria

With jogging a national obsession, we must mention march hemoglobinuria, a transient intravascular hemolysis with hemoglobinuria following strenuous running or long-distance walking. The disorder is caused by pounding the soles of the feet against a hard surface, with mechanical trauma to the red cells. Hemoglobinuria clears six to twelve hours after exercise, and the condition is benign, though frightening. Treatment consists of retraining the runner to alter his pounding gait, adding padded insoles to the shoes, or switching to bird watching. (Too much bongo drumming can also result in hemoglobinuria.)

C. Summary

In summary, the red cell membrane is a very complex structure that evolved in response to the need for increased oxygen carrying capacity. Many different abnormalities of this membrane exist, some hereditary, some acquired. All may result in premature destruction of red cells with subsequent anemia.

VIII. Red Cell Metabolism

The red cell is the only cell in the body with no nucleus and no mitochondria. It functions for four months without new protein synthesis or a Krebs cycle. It metabolizes glucose, its basic fuel, to maintain its osmotic equilibrium, regulate the position of its oxygen dissociation curve, keep iron in the ferrous state, and prevent denaturation of its proteins and lipids. Here we will review the red cell's metabolic features, examine a very common enzymatic abnormality, and explore the way in which the red cell regulates oxygen delivery.

Glucose enters the red cell by a facilitated transport system that is independent of insulin. The hexokinase reaction phosphorylates glucose at the sixth carbon atom. Glucose-6-phosphate is then metabolized in the red cell by both the glycolytic pathway and the pentose phosphate pathway, also known as the hexose monophosphate shunt or HMP (Fig. 4.11).

A. Glycolytic Pathway

The glycolytic pathway uses glucose to generate three important end products: ATP, 2,3-DPG, and NADH.

- **ATP**

Figure 4.11 shows the glycolytic (Embden-Meyerhof) pathway, which generates 2 moles of ATP per mole of glucose consumed. ATP is used by the erythrocyte to power the ATPase sodium-potassium pump that maintains the erythrocyte membrane in a flexible, highly deformable state. **When ATP production is inadequate, the erythrocyte loses potassium and gains sodium and calcium. The membrane becomes so stiff that it no longer can squeeze through pores in the spleen.**

- **2,3-DPG**

Figure 4.11 shows that the glycolytic intermediate 1,3-DPG (BPG) may alternatively pass through a side pathway known as the "Rapaport-Luebering shunt," in which 2,3-diphosphoglycerate (2,3-DPG or BPG) is formed. When this occurs, there is no net synthesis of ATP, but **2,3-DPG which is made instead, plays a critical role in regulating the position of the oxygen dissociation curve thus influencing hemoglobin function and oxygen transport.**

- **NADH**

The function of red cells is to bring high concentrations of oxygen to the tissues. Oxygen is a very reactive molecule that oxidizes the very hemoglobin that transports it at a rate of 1-3% per day. When the iron in hemoglobin is oxidized it is called methemoglobin (Fe^{+++}). Methemoglobin does not deliver oxygen, because hemoglobin that contains only ferric iron does not bind oxygen, while hemoglobin that contains some ferric and some ferrous iron has a left-shifted oxygen dissociation curve and will not release bound oxygen in tissues. Thus a mechanism must exist to continuously reduce methemoglobin (Fe^{+++}) back to hemoglobin (Fe^{++}). The third end-product of the glycolytic pathway, NADH, donates its electron via the enzyme methemoglobin reductase for this purpose (Fig. 4.11).

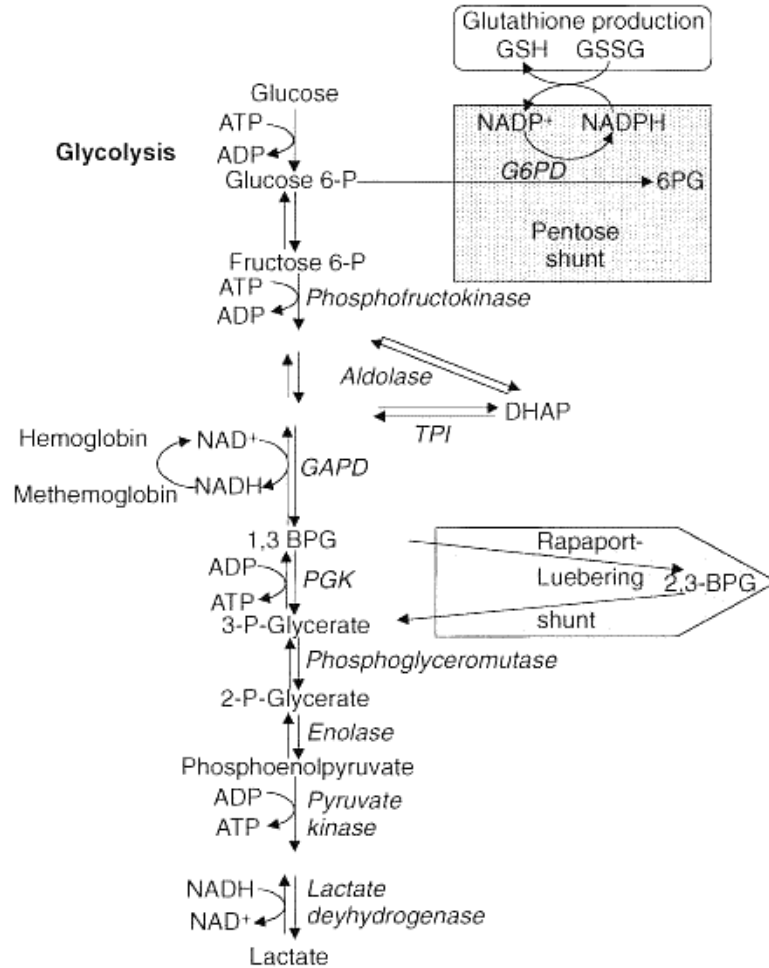


Figure 4.11. **Glycolytic, pentose phosphate, and Rapaport-Leubering pathways in the red cell.** (From MD Consult)

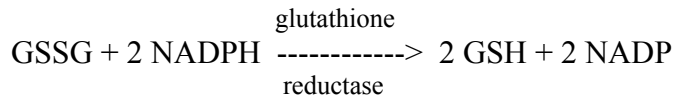
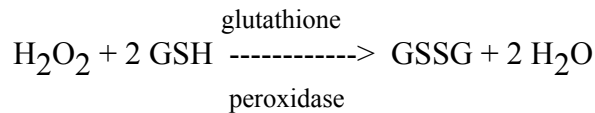
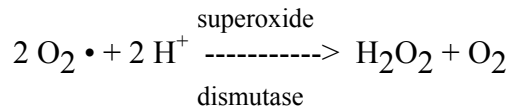
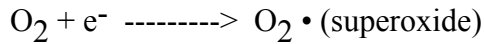
B. Pentose Phosphate Pathway

The major function of the pentose phosphate pathway is to protect the red cell membrane and globin chains from direct damage by oxygen radicals. It does so by producing NADPH.

Under normal circumstances, approximately 95% of glucose is metabolized through the glycolytic pathway; the remaining 5% is metabolized in the pentose phosphate pathway (Fig. 4.11). In this pathway, glucose-6-phosphate is first converted to 6-phosphogluconate (6PG) by the enzyme glucose-6-phosphate dehydrogenase (G6PD). In the process, NADP is reduced to NADPH.

NADPH in turn serves as an electron donor for the reduction of oxidized glutathione (GSSG). Reduced glutathione (GSH) is present in red cells in high concentration. It serves the critical function of protecting proteins and lipids of the red cell from oxidant denaturation.

Oxidants (superoxide and hydrogen peroxide) are common products of biological processes. They can result from normal oxidation reactions (including the reactions of molecular oxygen with hemoglobin), drug metabolism, ionizing irradiation, and the killing of bacteria by leukocytes. If unopposed, these reactive species will attack hemoglobin, enzymes, and cell membranes, causing irreversible damage. As shown in the following series of reactions, **glutathione reduces peroxides to water, thus protecting cellular constituents.**



When the oxidation product GSSG is reduced, NADPH is depleted. This temporarily increases the flow of glucose-6-phosphate into the pentose phosphate pathway to replenish NADPH. NADPH also reduces catalase, another important red cell enzyme that breaks down hydrogen peroxide. As discussed in subsequent paragraphs, these protective mechanisms are jeopardized by enzyme deficiencies in the pentose phosphate pathway.

FYI

Although NADH from the glycolytic pathway cannot substitute for NADPH to reduce glutathione, NADPH produced by the pentose phosphate pathway can be harnessed to substitute for NADH to reduce methemoglobin. This will occur only if an intermediate electron acceptor is given to the patient. The best known drug for this purpose is methylene blue, which is used to treat chemically-induced methemoglobinemia.

IX. Deficiencies and Defects

Enzyme Deficiencies of the Glycolytic Pathway

A. Pyruvate kinase (PK) deficiency

The most common **glycolytic** enzyme defect and the second most common erythrocyte enzyme deficiency is pyruvate kinase (PK) deficiency. This enzyme catalyzes the conversion of 2-phosphoenolpyruvate to pyruvate with production of ATP. Its deficiency causes subnormal ATP production. The decrease in ATP synthesis adversely affects cation transport, causing PK-deficient red cells to lose potassium and gain sodium at an accelerated rate, resulting in rigidity of the red cell membrane and extravascular hemolysis in the spleen.

The block at this point in the glycolytic pathway leads to **increased** concentrations of intermediates proximal to the block, especially 2,3-DPG (Fig 4.11). The high level of 2,3-DPG markedly decreases the affinity of hemoglobin for oxygen, facilitating oxygen delivery. Patients with PK deficiency therefore have greater tolerance of anemia than do persons with other anemias of equal severity.

PK deficiency is an autosomal recessive trait. It is described mostly in northern Europeans. The primary clinical manifestation in homozygotes is chronic hemolytic anemia. In most patients, anemia, jaundice, or both are noted in infancy or in early childhood. In severe cases, hemolysis may produce neonatal jaundice, requiring exchange transfusion. As in other chronic hemolytic

FYI

Other glycolytic enzyme deficiencies

An inherited deficiency of NADH-methemoglobin reductase, the enzyme that reduces NAD to NADH, causes congenital methemoglobinemia, but not hemolytic anemia. This enzyme is also low in normal neonates, rendering newborns susceptible to the effects of drugs and toxins that produce methemoglobinemia. One such natural toxin is nitrate in well-water, which is converted to nitrite in the gut.

anemias, transient aplastic crises—the result of parvovirus B19 infection—may be observed.

B. Defects of the Pentose Phosphate Pathway

Glucose-6-phosphate dehydrogenase (G6PD) deficiency is the most common red cell enzyme disorder, with an estimated 400 million affected individuals worldwide. The incidence is especially high in people with roots in Africa, the Mediterranean basin, and the Middle East. 30% of Sardinians, 60% of Iraqi Jewish Kurds, and 12% of African American men carry the gene. Why so prevalent? Along with sickle trait, thalassemia trait, hemoglobin E, and hereditary elliptocytosis, it offers some protection against falciparum malaria.

The gene for G6PD is on the X chromosome. Males, therefore, are hemizygotes and more likely than women to have clinical disease. In females, one of the two X chromosomes is randomly inactivated in each stem cell at the 6 cell stage of embryonic life, resulting in mosaicism (Fig 4.12). If, by chance, most of the healthy G6PD X chromosomes are inactivated, even a heterozygote female may have very low G6PD levels.

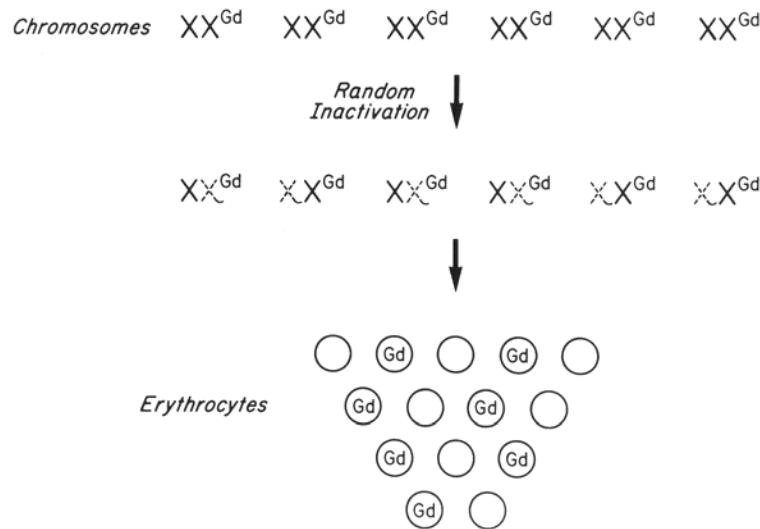


Fig 4.12 Random inactivation of the X chromosome during fetal life

1. G6PD Variants

Electrophoretic population screening has identified over 400 different G6PD variants, most of which function perfectly normally. They are subdivided into several classes as follows:

- **Variants with normal function**

G6PD B is the normal wild type enzyme. G6PD A, one of the many normally functioning variants, is found in 20-40% of people of African descent. Although it causes no hemolysis, G6PD A has been very valuable in validating the monoclonality of malignant disease. Due to X chromosome inactivation in early fetal life, a woman who is heterozygous for G6PD A and G6PD B should have G6PD A in about half of her body cells and G6PD B in the other half. When a malignancy develops in such a patient, all the tumor cells contain either G6PD A or G6PD B, but not both. **This helps prove that the cancer arose from a single cell.**

- **Variants that cause chronic congenital hemolytic anemia**

This is the rarest form of disease. Most are associated with amino acid substitutions at the site where G6PD attaches to NADP. Patients are often identified in the neonatal period when they become jaundiced and may require exchange transfusion.

- **Variants that cause hemolysis only with oxidant stress**

This constitutes the bulk of patients with clinically significant G6PD deficiency. Most often the stress is infection, either bacterial or viral. The presumed mechanism is generation of superoxides and hydrogen peroxide by neutrophils and macrophages. Oxidant drugs, diabetic ketoacidosis, and the newborn period are other precipitating events. Between attacks, the hemoglobin is normal.

The oxidant stress induced variants come in 3 forms:

- **Mild:**

The prototype is G6PD A⁻, a mutation of the normally functioning G6PD A seen in people of African descent. The A⁻ enzyme has a half-life of 13 days compared with the normal G6PD B half-life of 60 days. When stressed, therefore, only the older red cells hemolyze. Newly-made reticulocytes and younger red cells survive. A new steady state is reached and the anemia improves even if the oxidant stress continues. This is the variant seen in about 12% of African American men.

- **Severe:**

The prototype is G6PD Mediterranean. Here the half-life of the enzyme is measured in hours. It is virtually undetectable even in reticulocytes. When subjected to oxidant stress, severe intravascular hemolysis with profound anemia, hemoglobinuria, and jaundice may occur. As the name implies, this is the common variant in the Mediterranean region. As in the mild types, infection is the most common precipitating event and the hematocrit is normal between episodes.

FYI

Favism

There is a subset of patients with G6PD Mediterranean whose hemolysis may also be triggered by inhaling the pollen of blooming fava bean plants or eating raw fava beans, a popular food in the Mediterranean and Middle East. Small children are most likely to be affected. It is believed that a second hereditary defect is required in which an ingredient of the beans is aberrantly metabolized to an active oxidant. Massive, life-threatening hemolysis may occur.

2. Drug-induced hemolysis

A number of oxidant drugs may trigger hemolysis in G6PD deficient patients. The enzyme defect was actually discovered during WWII when about 10% of African American soldiers developed hemolysis after taking the antimalarial Primaquine.

The drugs in wide current clinical use that may cause hemolysis in patients with either G6PD A⁻ or G6PD Mediterranean are:

- **Sulfamethoxazole**, the sulfa moiety in the antibiotic TMP/Sulfa.
- **Nitrofurantoin**, an antibiotic sometimes used for treating urinary tract infections.
- **Primaquine**, occasionally used to treat malaria or pneumocystis pneumonia.
- **Dapsone**, used for treating leprosy and some skin diseases.

A number of other drugs are probably not safe for patients with the congenital chronic hemolytic anemia variants. On the other hand, many drugs previously thought to be contraindicated, such as aspirin and acetaminophen, have been exonerated.

It must be said that the vast majority of patients with G6PD deficiency, including those with G6PD A⁻ and G6PD Mediterranean go through life without ever having an episode of hemolysis.

3. Pathophysiology of the hemolysis:

When red cell levels of G6PD are low, the pentose phosphate shunt cannot generate enough NADPH to reduce glutathione and catalase in the face of oxidant stress. Unopposed hydrogen peroxide and superoxide then oxidize globin and membrane SH groups. Oxidized globin chains precipitate and form disulfide bridges to the red cell membrane, rendering the cell undeformable. These precipitated globin chains are known as Heinz bodies. The rigid red cells are then phagocytized by splenic macrophages.

If oxidant stress is severe, membrane SH groups also oxidize, creating small membrane holes and intravascular hemolysis. Thus both intra and extravascular hemolysis may occur simultaneously.

4. Diagnosis:

G6PD activity in red cells may be measured directly, but in patients suspected of having the A⁻ variety, this should not be done immediately after a hemolytic episode because the unhemolyzed young cells have G6PD levels that are close to normal. Testing should be delayed for several weeks. The Mediterranean variant always has very low levels of red cell G6PD and testing may be done at any time.

X. Hemoglobin Function, 2,3-Diphosphoglycerate, and Regulation of Oxygen Release

A. Molecular Physiology

Normal adult hemoglobin contains four globin chains, two alpha and two beta. A heme group is attached at a specific site on each globin chain. One hemoglobin molecule combines reversibly with four molecules of oxygen to form oxyhemoglobin. (Fig 4.13)

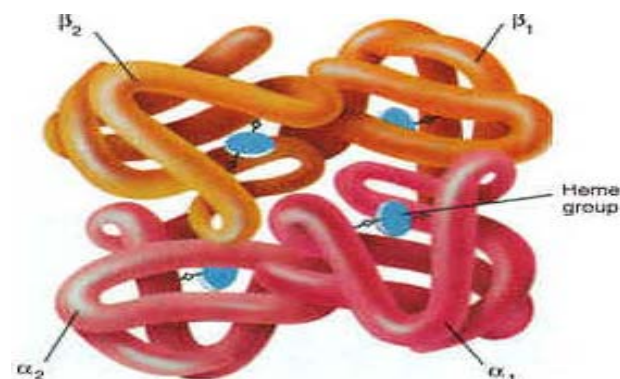


Fig 4.13 Hemoglobin molecule

A plot of the partial pressure of oxygen against the percentage of hemoglobin saturated with oxygen is sigmoid in shape (Fig. 4.14). This shape is of great physiologic importance. Its upper flat portion allows the blood to become virtually saturated with oxygen at the PO_2 in the pulmonary alveoli. Its steep down slope permits release of a great deal of oxygen with only a modest drop in PO_2 as blood flows through tissue. The sigmoid shape of the oxygen equilibrium curve is a consequence of "heme-heme interaction," or "subunit cooperativity," with oxygen binding to the first heme increasing the strength of its binding by the second, the second increasing the strength of binding by the third, and so on. This characteristic of hemoglobin depends upon its tetrameric structure and on the presence of dissimilar chains in the tetramer (two alpha and two beta chains in the case of normal adult human hemoglobin). Thus, monomers such as myoglobin yield an oxygen equilibrium curve that is a rectangular hyperbola; as does a tetramer containing only beta chains (β_4 = Hemoglobin H).

An elegant explanation has been devised by Perutz to account for this shape of the oxygen equilibrium curve. There are ionic bonds within and between the alpha and beta chains, which stabilize the molecule. These bonds are broken sequentially as each heme group is oxygenated. This causes the subunits to move significantly with respect to one another. Rupture of salt bonds also causes subtle movement within the adjacent subunit such that the affinity of its iron for oxygen increases. The hemoglobin molecule, like the lung, thus "breathes" as oxygen comes and goes (Fig. 4.15.).

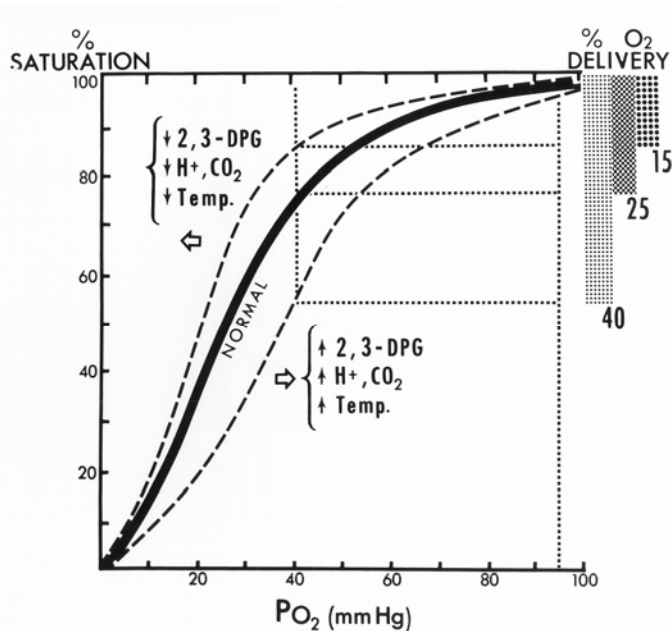


Figure 4.14. The normal blood-oxygen equilibrium curve (middle curve) and left- and right-shifted curves. At a given PO_2 (40 mm Hg in this example), the percentage of oxygen released (bars at right) varies appreciably. (From Harkness DR: *Hereditary disorders of red cells, in Fundamentals and Clinical Aspects of Internal Medicine*, Miami, University of Miami).

The middle curve of Fig. 4.14 depicts the normal blood-oxygen equilibrium curve. The PO_2 at 50% saturation is termed the "P50"; its average value is 27 mm Hg in normal subjects under standard conditions. When shifted to the right, blood-oxygen affinity is reduced, P50 is higher, and more oxygen is released from blood at any given pressure. The opposite is true when the curve is shifted to the left; blood PO_2 must fall farther for a given amount of oxygen to be released.

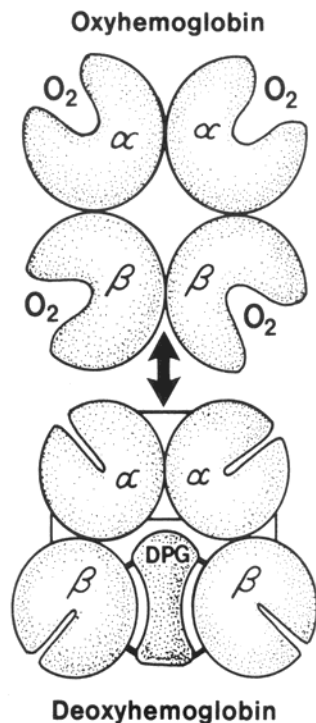


Figure 4.15. In the deoxygenated state, the hemoglobin molecule opens to accept a single 2,3-DPG, and hydrogen ions establish salt bridges between individual chains. With oxygen uptake, the salt bridges are ruptured, 2,3-DPG and CO₂ expressed, and the heme groups open to receive molecules of oxygen. (From Hillman, Ault. *Hematology in Clinical Practice*, McGraw Hill, NY)

Several factors normally regulate the position of the oxygen equilibrium curve. These are pH, PCO₂, temperature and 2,3-DPG.

B. Bohr Effect

In 1906 Christian Bohr discovered that hemoglobin-oxygen equilibrium was sensitive to the partial pressure of carbon dioxide in blood. The effect of carbon dioxide is largely due to the associated decrease in pH. Acidification of blood shifts the oxygen equilibrium curve to the right, increasing oxygen delivery to the tissues.

C. Carbon Dioxide and Temperature

Carbon dioxide also has a smaller direct effect on the hemoglobin-oxygen equilibrium. In addition to its effect on pH, carbon dioxide binds directly to the N-termini of alpha and beta chains of hemoglobin, and this binding produces a right shift of the curve. Increased temperature also shifts the curve to the right.

These effects of pH, PCO₂, and temperature on the oxygen-binding curve are biologically important. For example, during vigorous exercise, muscles produce lactic acid, carbon dioxide, and heat, all of which enhance oxygen release at the site where the oxygen is needed. On return to the lung, PCO₂ falls, pH rises, and the blood is cooled, all of which promote oxygen binding by hemoglobin.

D. 2,3-DPG

In 1925, an organic phosphate, 2,3-diphosphoglycerate (2,3-DPG), was found in high concentration in red cells. The function of 2,3-DPG remained unknown for forty years. In 1967 it was discovered that 2,3-DPG has a profound effect on the binding of oxygen by hemoglobin. By decreasing hemoglobin-oxygen affinity, it facilitates the release of oxygen from hemoglobin to the tissues. The 2,3-DPG concentration in normal human erythrocytes is about 4.5 mM—nearly equimolar with hemoglobin. The oxygen equilibrium curve shifts progressively to the right as erythrocytic 2,3-DPG concentration rises.

2,3-DPG exerts its effect by combining with deoxyhemoglobin and appreciably lowering the oxygen affinity (raising the P50, see Fig. 4.15). With oxygenation, 2,3-DPG is expelled. Thus, 2,3-DPG dissociates from and reassociates with hemoglobin during oxygenation and deoxygenation:



The gamma chains of fetal hemoglobin ($\alpha_2\gamma_2$) do not bind 2,3-DPG. This explains why the oxygen equilibrium curve of the fetus and neonate lies to the left of the adult curve despite similar 2,3-DPG levels.

Conditions associated with changes in 2,3 DPG

Anemia or any cause of hypoxia such as lung disease, heart failure, and high altitude increases the amount of deoxygenated hemoglobin in the red cell. This stimulates synthesis of 2,3-DPG, an important compensatory mechanism that results in better oxygen delivery to the tissues. High serum phosphate levels also promote 2,3-DPG synthesis and cause a right shift. This may explain the lower hemoglobin levels seen in children as they have higher phosphate levels than adults.

E. Fetal Blood Oxygen Affinity

A left shift of the blood-oxygen equilibrium curve is present in the fetus of virtually every species. This allows the fetal blood to bind more oxygen in the placenta, where PO_2 is lower than it is in the lung. In humans, this is accomplished by fetal (F) hemoglobin, which has a low affinity for 2,3-DPG. Other mechanisms for increasing oxygen affinity are employed in other species.

XI. Acquired Abnormalities of Hemoglobin

Carboxyhemoglobin

Carboxyhemoglobin is the name given to hemoglobin that is combined with carbon monoxide. A small amount of carbon monoxide is produced endogenously as a result of heme catabolism. This generates a carboxyhemoglobin level of about 0.4% (of total hemoglobin), though normal individuals usually have a level of 0.5% to 1% as a result of low level environmental exposure. Higher levels most often result from smoking (2% to 15%) or exposure to incompletely burned carbon or hydrocarbon fuels. Carboxyhemoglobin imparts a bright red color to the blood and a bright, cherry-red color to the skin and mucous membranes of patients with carbon monoxide

poisoning. Common manifestations are shown in Table 4.2. It is noteworthy that even a modest increase in carboxyhemoglobin due to cigarette smoke or heavy traffic causes subtle changes in neurologic and intellectual function.

The major effect of carbon monoxide is disruption of oxygen transport due to displacement of oxygen from hemoglobin. Significant binding of carbon monoxide to hemoglobin occurs at low PCO values, since the affinity of hemoglobin for carbon monoxide is 236 times greater than its affinity for oxygen.

Table 4.2
ACUTE TOXICITY OF CARBON MONOXIDE

| Carboxyhemoglobin Saturation (%) | Effect |
|---|---|
| 3 – 10 | subtle alteration of vision, hearing and response times |
| 10 – 20 | headache, exertional dyspnea |
| 20 – 40 | fatigue, altered judgment, dizziness |
| 40 – 60 | confusion, collapse |
| > 60 | coma, convulsions, death |

It is well known that a reduction in blood oxygen capacity due to anemia is far better tolerated than an equivalent reduction due to carbon monoxide. This is because the binding of carbon monoxide shifts the oxygen equilibrium curve of the remaining non-carbon-monoxide-liganded hemoglobin appreciably to the left. (Fig 4.16). This significantly lowers the release of oxygen in the tissues.

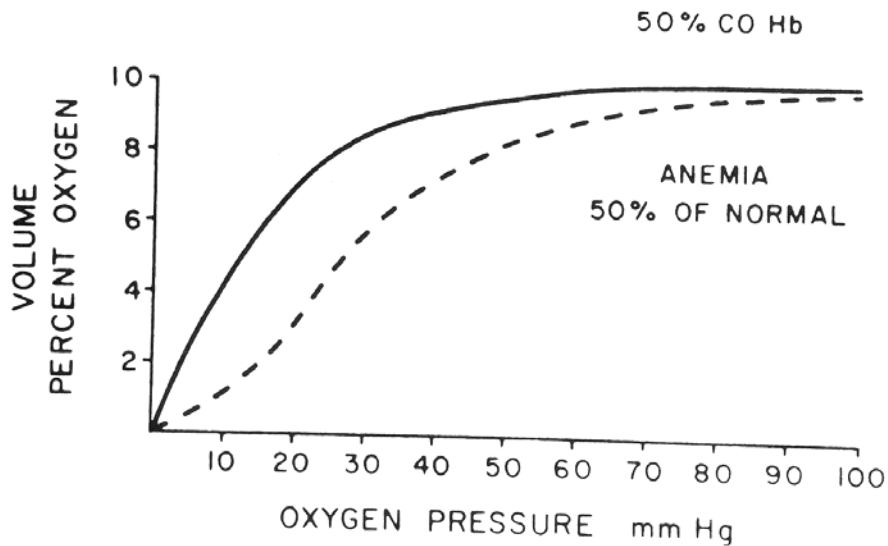


Figure 4.16. Effect of carbon monoxide on the position of the oxygen equilibrium curve in comparison to anemia. In both instances, the oxygen content of arterial blood is reduced by 50%, but much more oxygen can be released at a given tissue PO_2 in anemia.

FYI

Methemoglobin

Methemoglobin refers to hemoglobin in which the iron has been oxidized to the ferric state. This occurs to the extent of one to three percent daily, and small amounts of methemoglobin (less than one percent) are present normally. Increases in methemoglobin may occur as a result of

1. A hemoglobinopathy (hemoglobin mutation) called Hemoglobin M,
2. NADH methemoglobin reductase deficiency
3. Exposure to drugs and other chemicals.

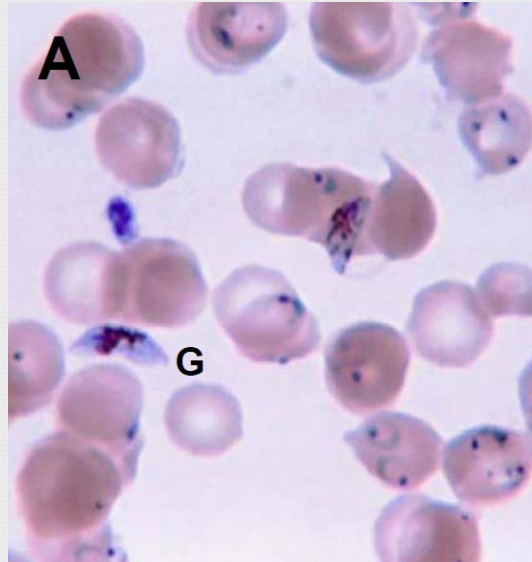
Toxins do not cause gross methemoglobinemia until the capacity of the NADH-methemoglobin reductase system is exceeded. This is more likely to occur in babies, due to the low activity of the enzyme in infancy, or in persons heterozygous for methemoglobin reductase deficiency. Homozygous methemoglobin reductase deficiency would be a lethal mutation.

The effect of methemoglobin on oxygen delivery is similar to that of carboxyhemoglobin: The oxygen binding capacity is reduced and the oxygen equilibrium curve of the remaining normal hemoglobin is shifted to the left. Symptoms, however, are milder and do not occur until the concentration reaches about 30%. Methemoglobin turns blood brown.

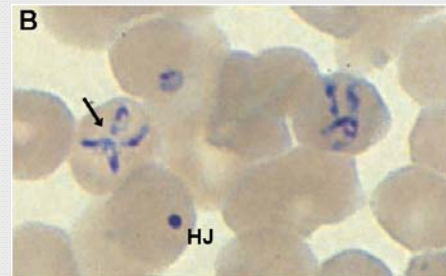
FYI

Hemolysis also can be caused by direct infection of RBC by parasites such as *Babesia* and the malarial organisms of *Plasmodia*. In both cases, the merozoite forms of the organism infect RBC where they replicate and mature from “ring form” trophozoites into merozoites or schizonts and literally break open the RBC to release more infectious merozoites. With higher infectious burdens, this can lead to hemoglobinuria, fever, and more severe symptoms including death.

Although it is no longer endemic in the U.S., malaria affects 300 million people worldwide and kills 3 million a year, primarily in a belt around the equator that includes Central America and the Caribbean. Indeed it is the major evolutionary force behind the emergence of the hemoglobin and enzyme variants that we discuss in the course, whose presence makes RBC less hospitable to the *Plasmodium* parasite. There are four major *Plasmodia* species that infect humans, including *P. malariae*, *P. ovale*, *P. vivax*, and the more dangerous *P. falciparum*, which is now resistant to chloroquine in much of the world. The 48 hour replication cycle (72 in *P. malariae*) within the RBC of *Plasmodia* accounts for the regular relapsing fever pattern seen in malaria as billions of schizonts burst from the host RBC. On the peripheral blood smear, the trophozoites appear as rings with dark blue chromatin dots, particularly in *P. falciparum* infection (image A). Sometimes, the gametocyte forms can be seen, with *falciparum* having a characteristic crescent shape (marked G in image) while other species are more round. In most patients, infected RBC are much rarer than seen here.



Babesiosis is a tick-borne disease (the Ixodes deer tick that also carries Lyme Disease causing *Borrelia*) endemic in the northeast and upper Midwest regions of the U.S., as well as in Europe. *Babesia* trophozoites mimic malaria to some extent with irregular ring-like conformations. Classically, the merozoites form four-sided “Maltese crosses” as seen in image B. This patient was asplenic as evidenced by the presence of a Howell-Jolly body (HJ) in one of the red cells. Asplenic and immunosuppressed patients are most at risk for severe *Babesia* infections.



XII. Summary

Although the red cell lacks a nucleus and mitochondria, it is a metabolically active cell with a glycolytic pathway and a pentose phosphate shunt. Deficiencies of critical enzymes in these pathways result in chronic or drug-induced hemolytic anemia. X-linked G6PD deficiency is the most common red cell enzyme deficiency world-wide. Various factors also affect the binding of oxygen to hemoglobin, including the presence of fetal hemoglobin, pH (Bohr effect), temperature, exposure to carbon monoxide, and agents that change the oxidation state of iron.

CHAPTER 5

DISORDERS OF GLOBIN SYNTHESIS

Key Concepts:

- Pathophysiology, epidemiology, and major clinical and laboratory features of sickle cell disease
- Pathophysiology, epidemiology clinical and laboratory features of the various forms of alpha and beta-thalassemia

Learning Objectives:

1. List the protein chains (globins) found in hemoglobin and describe how these change during fetal development and infancy.
2. Be able to diagnose sickle cell disease using clinical and laboratory data, and describe its molecular basis, genetics, demographics, pathophysiology and major complications.
3. Be able to diagnose the various forms of thalassemia using clinical and laboratory data, and describe the molecular basis, genetics, demographics, pathophysiology, and major complications of these disorders.

I. Normal Hemoglobin

Normal adult hemoglobin is called Hemoglobin A (HbA). It is composed of 4 globin chains – 2 alpha (α) and 2 beta (β) chains. Each globin chain is complexed to a heme group. Thus, 4 globin chains and 4 heme groups comprise the complete hemoglobin molecule (Fig. 4.13). There are also 2 minor adult hemoglobins: hemoglobin F (fetal hemoglobin), which has 2 α and 2 gamma (γ) chains, and hemoglobin A₂, which has 2 α and 2 delta (δ) chains. **The four globin chains in each hemoglobin molecule bind oxygen in a cooperative fashion, which accounts for the sigmoid shape of the oxygen dissociation curve** that permits acquisition of oxygen in the lungs and efficient unloading of oxygen in the tissues. (see Chapters 1 and 4)

A. Organization and Expression of Human Globin Genes

Two clusters of tightly linked globin genes exist on two different chromosomes. (Fig 5.1). **On chromosome 16, there are 2 α genes** and an embryonic ζ (zeta) gene. **On chromosome 11, there is a β gene and a δ gene, 2 γ (fetal) genes (G and A),** and two globin genes only expressed in during embryonic life. **Thus in diploid cells, there are normally 4 α genes, 2 β and 2 δ genes, and 4 γ genes.**

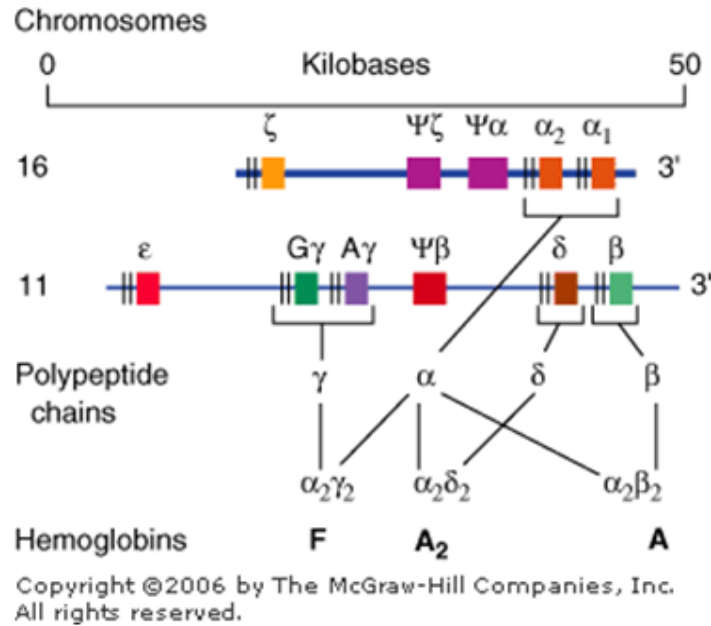


Fig 5.1. The human hemoglobins in the fetus, and adult. (From Weatheral DJ, Clef JB: *The Thalassemia Syndromes*, St. Louis, Blackwell, p. 29).

α =alpha β =beta γ =gamma δ =delta ϵ =epsilon ζ =zeta

B. Fetal Hemoglobin

Fetal hemoglobin (HbF: $\alpha_2\gamma_2$) is the primary hemoglobin in the fetus, whereas the primary hemoglobin after early infancy is HbA ($\alpha_2\beta_2$). The switch from γ chain production to β chain production (i.e., from HbF to HbA) occurs during the first six months of life as the bone marrow replaces the liver as the major site of hematopoietic activity. Adults continue to produce a little HbF. Delta chains (δ) are also produced at low level and combine with α chains to form small amounts of HbA₂ ($\alpha_2\delta_2$). (**Fig 5.2**)

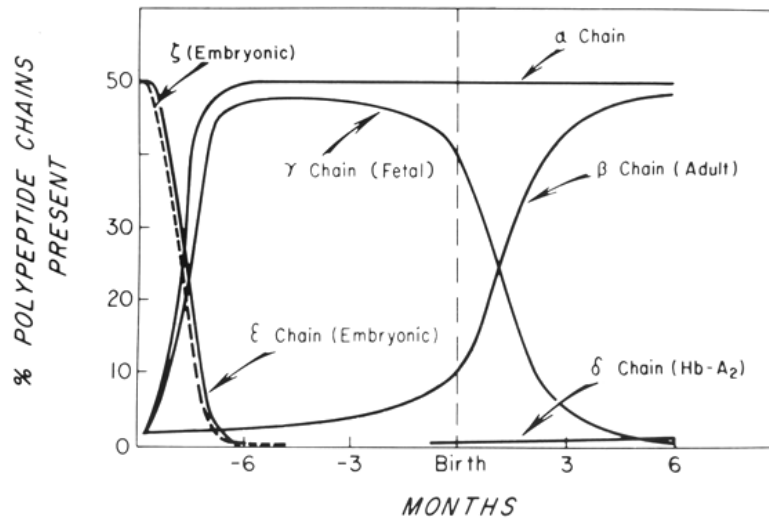


Fig. 5.2 Changes in human globin synthesis during prenatal and neonatal development. From Bunn, FH, Forget BG, Ranney, HM: *Human Hemoglobins*. Philadelphia, Saunders, p. 107.

II. Hemoglobinopathies and Thalassemias

A. Definitions

1. Hemoglobinopathies

An inherited mutation of a globin gene that causes a **qualitative** abnormality of a globin chain is called a hemoglobinopathy. This is usually due to a single nucleotide change in the DNA leading to a single amino acid change in the globin chain. Among the consequences of such a change may be a hemoglobin that polymerizes, crystallizes, or denatures. Sick cell anemia is the most important member of this group.

2. Thalassemia syndromes

Inherited **quantitative** defects in globin synthesis that result in imbalanced production of globin subunits are called thalassemia syndromes. They are characterized by decreased production of either the α or the β globin chain and a relative excess of the unaffected chain. The globin chains produced by the mutated gene are normal, but too few in number. The excess unaffected globin chains may attach to and damage the red cell membrane leading to destruction of red cell precursors in the marrow (ineffective erythropoiesis) or peripheral blood (hemolysis). The lack of adequate globin chain production also leads to decreased hemoglobin production. The thalassemias are genetically heterogeneous. They may be caused by deletions of an entire globin gene or by transcription mutants, RNA processing mutants, splicing mutants etc.

FYI

Hemoglobinopathies and thalassemias are not mutually exclusive. There are some mutations (e.g., hemoglobin E and hemoglobin Constant Spring) that alter both the structure of the globin chain and the rate at which it is produced.

B. Evolution of Sickle Cell Anemia and Thalassemia Syndromes

The mutations responsible for sickle cell anemia and the thalassemia syndromes became prominent several thousand years ago when deforestation created stagnant pools of water, giving rise to endemic malaria in India, equatorial Africa, and the Middle East. These mutations eventually spread throughout the world, initially with the slave trade and later with immigration to central Europe and the Americas.

It appears that heterozygosity for HbS and thalassemia is partially protective against falciparum malaria. The reasons for this are not fully understood, but several potential mechanisms have been proposed:

FYI

Potential mechanisms for protective qualities of Hb S and thalassemia heterozygosity

- Cultured RBCs with increased Hb F retard plasmodium growth.
- The parasite cannot acquire sufficient nutrients from hemoglobin in thalassemic RBC.
- Increased antibody binding to thalassemic RBCs enhances removal of infected cells from the circulation.
- Hb S is poorly metabolized by the malaria parasite.
- Plasmodium infected cells sickle and are removed from the circulation.
- Sickle cells have decreased potassium, creating a more hostile environment for the parasite.
- The rigid sickled RBC inhibits parasite invasion.

C. Detection of Abnormal Hemoglobins

In the U.S., all newborns are routinely screened for abnormal hemoglobins by electrophoresis. Hemoglobin variants migrate with characteristic mobilities due to differences in charge at the pH of the electrophoretic media. (Fig 5.3)

FYI

Standard electrophoresis can miss the rapidly migrating and unstable gamma tetramer referred to as Barts hemoglobin (γ_4), which forms when there is decreased or absent α globin production. PCR amplification coupled with specific oligonucleotide hybridization and restriction enzyme analysis can be employed to detect such α chain abnormalities.

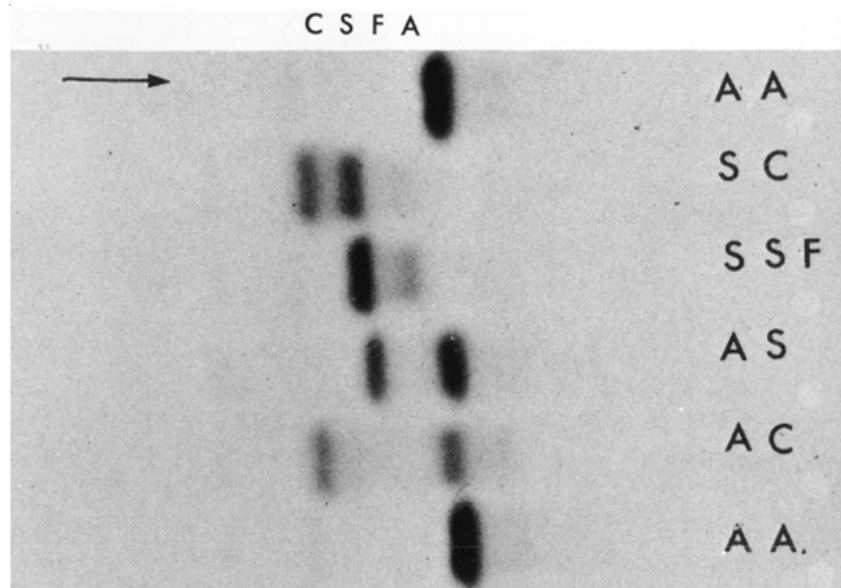


Fig. 5.3. Separation of various hemoglobins with electrophoresis on cellulose acetate, pH 8.6. Hemolysates represented are AA (normal adult), SC (hemoglobin SC disease), SSF (homozygous sickle disease, SS, with increased F), AS (sickle trait), and AC (C trait). (From Miale, JB: *Laboratory Medicine. Hematology*, St. Louis, Mosby, p. 857).

III. Sickle Cell Disease

A. Hemoglobin that Polymerizes: Hemoglobin S

The hemoglobin S abnormality provides one of the best examples of how an inherited single-point mutation in the DNA can lead to a devastating disease. **Sickle cell disease results from a specific mutation in the β globin gene. It is a chronic hemolytic anemia characterized by vasculopathy, vaso-occlusion, acute and chronic organ failure, and a shortened life expectancy. It is an autosomal recessive disorder.** The sickle phenotype occurs in individuals homozygous for β^S , and in those who are compound heterozygotes for β^S and certain other globin gene mutations (table 5.1). **The three most common genotypes that produce sickle disease are $\beta^S\beta^S$, $\beta^S\beta^0$ (compound heterozygosity for β^S and β^0 thalassemia), and $\beta^S\beta^C$ (compound heterozygosity for β^S and hemoglobin C). The heterozygote $\beta^A\beta^S$ (sickle trait) has little or no evidence of clinical disease.**

Table 5.1: Genetic and Laboratory Features of Some Common Sickle Hemoglobinopathies

| Genotype | Genetics | Prevalence among African-Americans | Hct (%) | MCV (fl) | HbS (%) | Hb A ₂ (%) | Relative severity (approx) |
|---------------------------|--|------------------------------------|---------|----------|---------|-----------------------|----------------------------|
| Normal (HbAA) | Homozygous HbAA | ----- | 38-50 | 80-95 | 0 | 2-3 | 0 |
| Sickle cell anemia (HbSS) | Homozygous SS | 1:600 | 18-28 | 85-95 | >85 | 2-3 | 4 |
| HbSC disease | Compound heterozygous HbS, HbC | 1:800 | 28-40 | 70-85 | 50 | 2-3 | 4 |
| HbS-β ⁰ -thal | Compound heterozygous HbS, Hb β ⁰ -thal | 1:1600 | 20-30 | 65-75 | >85 | 4-6 | 4 |
| HbS-β ⁺ -thal | Compound heterozygous HbS, Hb β ⁺ -thal | 1:1600 | 30-40 | 60-70 | 70-95 | 4-6 | 1-3 |
| Sickle cell trait (HbAS) | Heterozygous HbS | 1:12 | 38-50 | 80-95 | 35-40 | 2-3 | 0 |

Hb, hemoglobin; MCV, mean corpuscular volume

In sickle hemoglobin, a thymidine replaces an adenine in codon 6 of the β globin gene. The resultant hemoglobin S contains valine residues instead of glutamic acid at the 6th position of the β chain. This alters the physico-chemical properties of the hemoglobin molecule substantially, resulting in polymerization of hemoglobin when deoxygenated. As the HbS polymerizes, it distorts and dehydrates the red cell, causing it to assume the sickle shape. RBC membrane damage disrupts cation homeostasis, reducing the ability of the cell to maintain a normal K⁺ gradient.

HbS can resume its normal shape when it is reoxygenated, and so the sickling process is potentially reversible. However, with repeated sickling and unsickling, the cell accumulates enough membrane damage to become permanently sickled. These irreversibly sickled cells are apparent on the peripheral blood smear and have a short life span. Sickle cell disease is thus a form of hemolytic anemia.

There is heterogeneity among RBCs in sickle cell disease due to differences in the cellular level of fetal hemoglobin (HbF). HbF in a cell inhibits HbS polymerization, thus its quantity and its distribution in each cell will affect the likelihood of sickling. In general, patients who have more cells with relatively high HbF levels have less severe disease.

Sickle cells interact with endothelium and other blood cells to cause vaso-occlusion, particularly in the microvasculature. Hemolysis releases heme into the plasma and thereby consumes nitric oxide (NO). Hemolyzed RBCs also release arginase and destroy arginine, which is the precursor for NO. NO normally functions to relax smooth muscle and inhibit platelet aggregation and cell adhesion. Chronic depletion of NO in sickle cell disease causes vasoconstriction, increased

platelet aggregation, and endothelial activation, all of which contribute to the vasculopathy associated with the disease (**Fig 5.4**).

Sickling is highly dependent on the sickle hemoglobin concentration in the red cell, and is thus ameliorated by the presence of higher levels of HbF or, in the heterozygous state, HbA. Sickling is exacerbated by anything that increases the percentage of deoxyhemoglobin in the red cell, such as acidosis or increased 2,3 DPG, both of which shift the oxygen dissociation curve to the right.

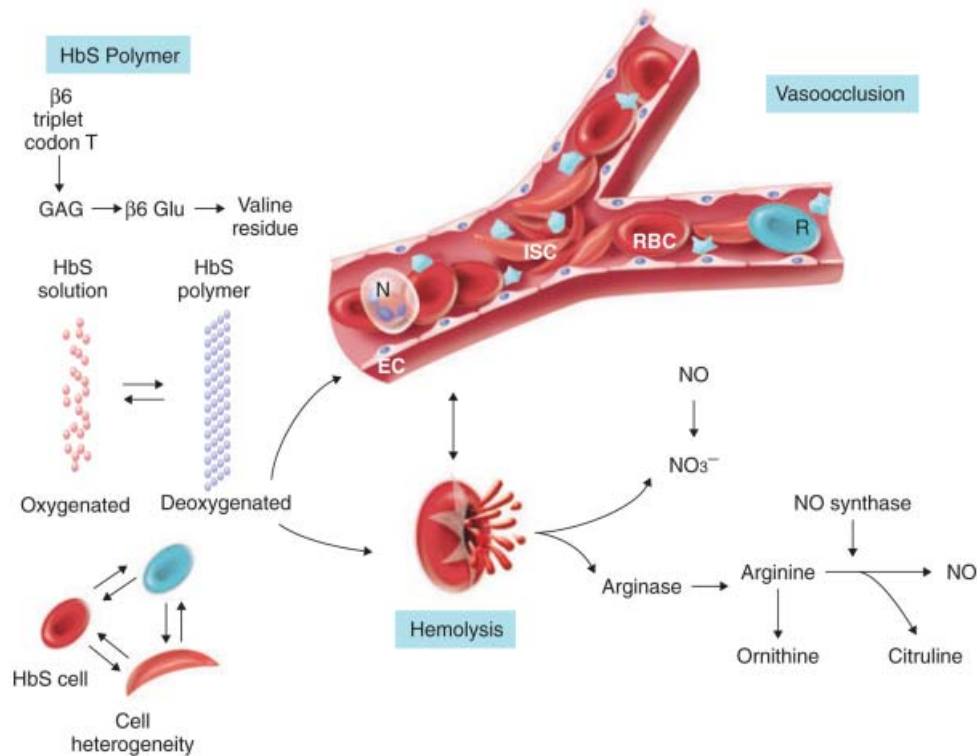


FIGURE 5.4 Pathophysiology of sickle cell disease. An adenine (A) to thymidine (T) transversion (A6T) at codon 6 in the β hemoglobin gene on chromosome 11 leads to the substitution of a glutamic acid codon by a valine codon. β^6 valine endows the hemoglobin S (HbS) molecule ($\alpha_2\beta_2^S$) with the property of polymerization when deoxygenated. HbS polymer injures the erythrocyte and leads to a heterogeneous population of sickle cells with damaged membrane cytoskeleton, reduced cation and water content, and altered distribution of membrane lipids. In the vasculature, sickle cells interact with endothelium and other blood cells to cause vaso-occlusion. Some damaged erythrocytes hemolyze intravascularly, thereby releasing heme into the plasma to scavenge nitric oxide (NO). Lysed erythrocytes also liberate arginase, which destroys L-arginine, the substrate for NO production, providing another mechanism for endothelial NO deficiency. The normal balance of vasoconstriction versus vasodilation is therefore skewed toward vasoconstriction, endothelial activation, and proliferation. EC = epithelial cell; ISC = irreversibly sickled cell; N = neutrophil; R = reticulocyte; RBC = red blood cell. (From Cecil – “*Pathophysiology of Sickle Cell Anemia*”)

B. Clinical Manifestations of Sickle Cell Disease:

Manifestations of sickle cell disease begin at about 6 months of age, when beta globin chain production becomes dominant and the levels of fetal hemoglobin decline.

Complications of sickle cell disease fall into the following categories:

- Hemolytic anemia and its consequences
- Consequences of vaso-occlusion
 - acute pain syndromes
 - acute organ damage
 - chronic organ damage
- Susceptibility to infection

1. Hemolytic Anemia and its consequences:

Sickled red cells are very undeformable and are unable to negotiate the small vessels in the liver and spleen. They are phagocytized by macrophages in both organs causing a chronic extravascular hemolytic anemia. Some intravascular hemolysis occurs as well. Most people with sickle cell anemia have hematocrits in the 25-30% range and sickle cells can be seen on peripheral smear (**fig 5.5**).

As with any hemolytic disorder, they have elevated reticulocyte counts, LDH, and unconjugated bilirubin. They are also prone to the complications of chronic hemolysis discussed in Chapter 4, including aplastic crises due to parvovirus infection, folate deficiency, bilirubin gallstones, and iron overload.

Life-threatening episodes of acute anemia may occur in children due to sequestration of red cells in a large spleen. These episodes dissipate with age as the spleen undergoes auto-infarction. After the spleen atrophies, the liver is the site of the ongoing chronic hemolysis.

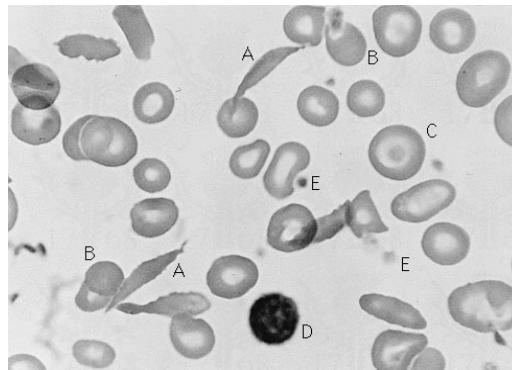


Fig 5.5 A= sickled cells
(www.britannica.com/EBchecked/topic/720818/blood-disease)

2. Vaso-occlusion

The sickle cell patient is subject to a lifelong, body-wide destructive process caused by occlusion of the microvasculature with ischemic death (infarction) of tissue. Microinfarctions lead to acute pain crises as well as to extensive acute and chronic organ damage. The deformed sickled cells sequester in the microvasculature, injuring and activating endothelial cells. Reticulocytes prematurely released from the marrow display

adhesive ligands that facilitate RBC-endothelial interactions. Sick cell patients with the highest rate of hemolysis have the highest reticulocyte counts and the highest vaso-occlusive risk. Neutrophils are elevated in most patients with sickle cell disease, an indicator of a chronic inflammatory state. Patients with higher neutrophil counts at baseline are at highest risk for serious vaso-occlusive complications such as stroke, acute chest syndrome, and priapism.

- **Painful crises**

Recurrent episodes of severe pain in bones, muscles, and abdomen are the hallmark of sickle cell anemia. The first manifestation may be painful swelling of the hands and feet called dactylitis. This starts at about 6 months of age, when HbF falls to adult levels. Pain crises frequently require hospitalization where rest, hydration, narcotics, NSAIDS, oxygen, and sometimes transfusion are given.

- **Acute organ damage**

- **Acute Chest Syndrome:**

About 20% of vaso-occlusive events progress to acute chest syndrome characterized by tachypnea, hypoxia, fever, pain, cough, and an infiltrate on chest x-ray. This is often associated with infection. This syndrome can be life-threatening and is treated with supplemental oxygen, transfusion or exchange transfusion, nitric oxide, bronchodilators, and antibiotics.

- **Stroke:**

This is a major complication of sickle cell anemia, with a risk of 11% by age 20, 15% by age 30, and 24% by age 45. Subclinical neurologic events and silent infarcts are even more common. The finding of increased blood turbulence on a cranial Doppler study identifies a child as being at high risk for stroke. Such individuals then undergo chronic transfusion therapy, which decreases the concentration of sickle cells and improves outcome.

- **Priapism:**

A prolonged painful penile erection can occur in up to 40% of men with sickle cell anemia. Venous flow is obstructed in the corpus cavernosa. Recurrent events can result in impotence.

- **Chronic organ damage**

Chronic ischemia and infarction may occur in almost any organ in the body. Leg ulcers, osteonecrosis of the femoral head, pulmonary hypertension, chronic kidney disease, heart failure, retinal disease, and increased fetal loss during pregnancy are among the more common manifestations.

3. Infection

Because of recurrent micro-occlusive events, the spleen atrophies in most sickle cell patients. This results in significant susceptibility to encapsulated organisms, especially the pneumococcus. Children are usually treated with penicillin prophylaxis from infancy until at least age 6. In addition, immunization to pneumococcus is recommended.

Infarcted tissue is particularly vulnerable to infection; this is a particular problem in bone, where Salmonella and Staphylococcal infections predominate.

C. Prevention and Treatment

Early diagnosis and care through a comprehensive sickle cell clinic is very helpful. A major therapeutic breakthrough in sickle cell disease has been the use of hydroxyurea. This agent can increase HbF production, decrease inflammation, affect cell-cell interactions, and improve the hydration status of the red cell. Children treated with hydroxyurea prior to autosplenectomy retain splenic function longer, show better growth curves, fewer vaso-occlusive events, and a slightly higher baseline Hb. In the adult population, hydroxyurea treatment has dramatically improved the quality of life for many patients.

Treatment with iron chelating agents (deferoxamine, given intravenously, or deferasirox, given orally) may decrease the incidence of end organ failure due to iron overload.

Prompt treatment of infection and painful crises is critical.

The only curative treatment for sickle cell anemia is allogeneic bone marrow transplant. This is not commonly performed due to a shortage of suitable donors and the potential morbidity associated with bone marrow transplant, which must be weighed against the somewhat unpredictable clinical course of sickle cell disease in an individual patient.

D. Sickle Trait

Sickle trait (HbA/S: $\beta^A\beta^S$) found in 10% of African-Americans, is basically asymptomatic and should not be considered a disease. However, because the renal medulla is hypoxic, acidotic, and hyperosmolar—all conditions that promote sickling—A/S patients may be unable to concentrate their urine and may have occasional episodes of painless hematuria. In special situations, such as severe dehydration or travel to very high altitudes, sickling may occur. Denial of employment or of life or health insurance because of sickle trait is not justified and is illegal in many states.

FYI

Other Hemoglobinopathies:

Worldwide, many different hemoglobinopathies have been identified by electrophoretic population studies. Most have no clinical manifestations. Some abnormal hemoglobins may:

- crystallize (hemoglobin C)
- cause decreased production of the β globin chain (hemoglobin E)
- denature (hemoglobin Köln)
- cause methemoglobinemia (hemoglobin M).
- alter O₂ affinity (hemoglobin Chesapeake)

1. Hemoglobin C – A crystallizing hemoglobin

In hemoglobin C, lysine rather than valine replaces glutamic acid in the 6th position of the beta chain. About 3% of African-Americans are A/C heterozygotes and one in 10,000 is a C/C homozygote. The C trait (A/C) is asymptomatic with a normal hemoglobin and a normal blood smear. Homozygosity results in a mild microcytic hemolytic anemia. The peripheral smear shows almost 100% target cells and rare hemoglobin crystals within the red cells. The shortened red cell life span is due to the crystals, which increase red cell rigidity and lead to phagocytosis by splenic macrophages. HbC is most important when combined with HbS in HbS/C disease. Red cells with compound heterozygosity ($\beta^S \beta^C$) do sickle but S/C disease is generally milder than S/S disease.

2. Hemoglobin E – Causes a decrease in β globin chain production.

HbE is an oddity. It is a hemoglobinopathy that behaves like a thalassemia. It is due to a single amino acid substitution of lysine for glutamic acid at the 27th position of the beta globin chain. Instead of polymerizing like sickle hemoglobin or crystallizing like hemoglobin C, this amino acid substitution decreases production of an otherwise normally functioning beta globin chain, i.e., it acts like a thalassemia. It is very common in Southeast Asia. Up to 40% of people from Laos are heterozygous. Like other hemoglobinopathies and thalassemias, it is thought to offer some protection against malaria. With increasing immigration from Southeast Asia, HbE is emerging as a significant concern in the United States. Heterozygotes and homozygotes have microcytosis and either mild or no anemia. HgE becomes very clinically significant when in the compound heterozygous state with beta thalassemia. These individuals often are transfusion-dependent.

3. Unstable hemoglobins (Prototype: Hb Köln)

Unstable hemoglobins (those that denature) are a rare cause of congenital hemolytic anemia. Mutations and deletions occur in the vicinity of the heme pocket, which disrupts the stability of the heme-globin linkage. Precipitation of the hemoglobin into 1-2 micron particles called Heinz bodies then occurs. The Heinz bodies attach to the red cell membrane, which results in decreased deformability and extravascular hemolysis. Hemolysis often increases in times of oxidative stress.

4. Hemoglobins that stabilize iron in the ferric⁺⁺⁺ state: Methemoglobinemia (Prototype: Hb M-Milwaukee)

Congenital methemoglobinemia, Hb with iron in the Fe⁺⁺⁺ state, is a very rare hemoglobinopathy that causes familial cyanosis. The patient appears blue and the blood is brown. The homozygous state has not been described and is presumed to be lethal, as Fe⁺⁺⁺ does not bind oxygen. The heterozygous state is benign, although the patient does present a disconcerting appearance. (Other causes of methemoglobinemia are discussed in Chapter 4. Other causes of cyanosis are discussed in your Heart and Lung courses)

5. Hemoglobins with altered affinity for oxygen: (Prototype: Hb Chesapeake)

Hb Chesapeake is a rare disorder in which the oxygen dissociation curve is shifted to the left, resulting in impaired oxygen delivery to tissues. To compensate, erythropoietin levels increase and hemoglobin levels rise. This erythrocytosis may result in hyperviscosity and an attendant risk of ischemic events and stroke. Phlebotomy may be required. Diagnosis is by measurement of hemoglobin oxygen affinity (P50), as the abnormal hemoglobin is difficult to distinguish from HbA by electrophoresis.

IV. Thalassemias

The term **thalassemia** refers to a **heterogenous group of inherited anemias characterized by decreased synthesis of one type of globin chain** (Fig. 5.6). Syndromes with decreased α and β chain production are referred to as α and β -thalassemia, respectively. Depending on the degree to which globin production is decreased, the clinical spectrum ranges from asymptomatic microcytosis and hypochromia to severe anemia leading to death *in utero*. The anemia of thalassemia is due to:

- ineffective erythropoiesis
- hemolysis
- decreased hemoglobin synthesis

The clinical manifestations are summarized in **tables 5.2 and 5.3**.

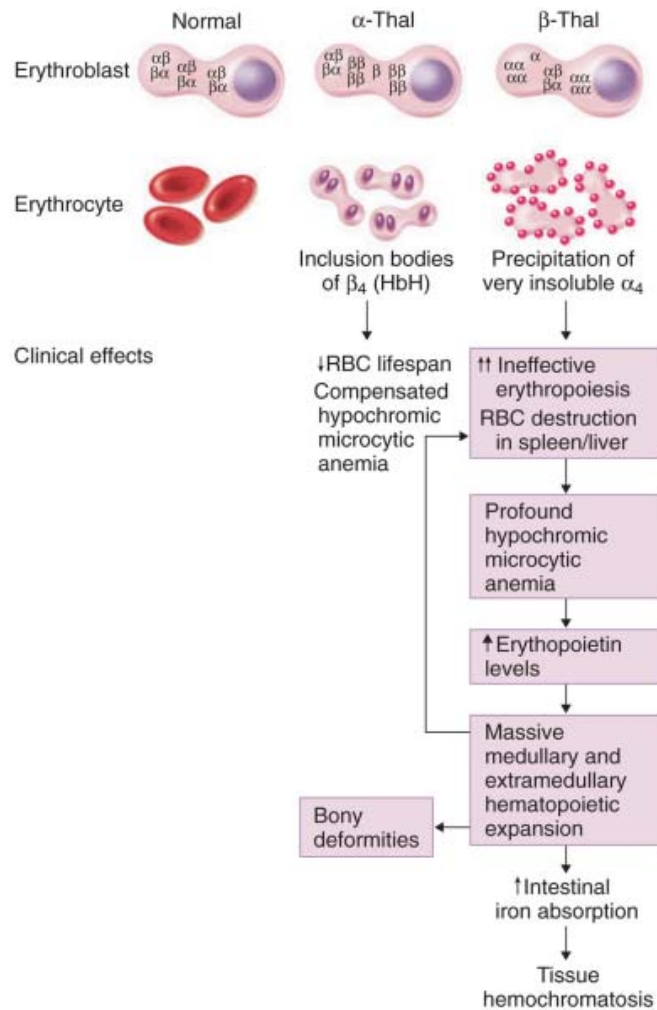


Fig 5.6 α - and β -thalassemia. Schematic representation of the pathophysiology of the clinically significant α - and β -thalassemia (Thal) syndromes. RBC = red blood cell.

Table 5.2 Clinical Classification of α -Thalassemias

| Classification | Genotypes | Severity | Treatment |
|---------------------|--|-------------------------------|---|
| α carrier | $-\alpha/\alpha\alpha$ | Silent | None |
| α Thal trait | $-\alpha/-\alpha$ $--/\alpha\alpha$ | Mild (microcytosis) | None (avoid unnecessary iron supplements) |
| HbH disease | $-\alpha/--$ | Moderate; hemolytic anemia | Folic acid, avoid oxidant drugs, transfusion and splenectomy as indicated |
| Hydrops fetalis | $--/--$ | Lethal <i>in utero</i> | Early referral to high-risk obstetric service |

Table 5.3 Clinical Classification of β -Thalassemias

| Classification | Genotypes | Severity | Treatment |
|--------------------------|---|---|---|
| β -Thal minor | β/β^0 β/β^+ | Mild (microcytosis with or without mild anemia) | None (avoid unnecessary iron supplements) |
| β -Thal intermedia | β^+/β^+ β^+/β^0 β/β^0 | Moderate-severe anemia | Folic acid, RBC transfusion and/or splenectomy as indicated |
| β -Thal major | β^0/β^0 | Severe, transfusion-dependent anemia | Early referral for allogeneic stem-cell transplant, if feasible; iron chelation; splenectomy if indicated |

Silent = normal or minimally abnormal hematology values; *mild* = hemoglobin level normal or slightly reduced with disproportionate microcytic hypochromic indices; *moderate* = hemolytic anemia, icterus, splenomegaly, although no regular transfusion requirement; *severe* = profound anemia with transfusion dependency, extramedullary hematopoiesis, growth retardation, bone abnormalities, hemosiderosis; *lethal* = death *in utero* from anemic congestive heart failure. The α -thalassemia syndromes usually result from deletions in one or more α -genes, indicated by the minus sign, or from mutations in the coding sequence. The β -thalassemia syndromes are typically the consequence of mutations that lead to a *decreased* level of normal β -chain production (β^+) or *absence* of β -chain production (β^0). Various combinations of these mutations give rise to syndromes of increasing severity. Note that identical genotypes (for example β/β^0) can produce variable phenotypes. (Adapted from Cecil *Textbook of Medicine*)

A. β Thalassemia Syndromes

These are extremely common genetic disorders in Asia, Africa, and the Mediterranean region. Many different mutations exist and the clinical phenotype is directly related to the effect of the mutation on the amount of β globin chain synthesis. Genes that produce no beta globin chain are called β^0 . Those that produce some beta globin chain are called β^+ . The amount of beta

globin chain produced depends on the specific mutation. Thus the phenotype is extremely heterogeneous, depending on whether the individual is a heterozygote or a homozygote and how much beta chain is produced by each affected gene. A convenient clinical classification system is based on a patient's baseline hemoglobin level.

1. β thalassemia major is defined as a Hb of < 6 g/dl. These patients are usually homozygous β^0/β^0 . No β globin is produced and α globin chains precipitate in the RBC, resulting in marked ineffective erythropoiesis and hemolysis. The only functional hemoglobins they have are HbF ($\alpha_2\gamma_2$) and HbA2 ($\alpha_2\delta_2$), and these are made in small amounts. This results in severe microcytic anemia, hepatosplenomegaly, and massive marrow expansion with attendant bony deformities. A major problem is iron overload due to the need for chronic red cell transfusions in conjunction with the increased iron absorption that accompanies ineffective erythropoiesis and hemolysis. Prior to the availability of iron chelating agents, these patients died prematurely from the effects of iron deposition in the heart, liver, and pancreas.

2. β thalassemia intermedia is defined as a Hb of 6-9 g/dl and may be due to any of the genetic combinations listed in table 5.3. Anemia is microcytic with target cells, nucleated RBC, and many other bizarre red cell forms on peripheral smear.

3. β thalassemia minor (Hb > 9 g/dl) patients are usually heterozygous β^+/β or β^0/β and are often completely asymptomatic. This disorder should be suspected in people with mild or no anemia but significant microcytosis who do not prove to have iron deficiency or the anemia of inflammation. It is important to recognize this entity in order to provide appropriate genetic counseling and to prevent a misdiagnosis of iron deficiency that might result in potentially harmful iron supplementation.

4. Diagnosis of β thalassemia:

There is no qualitatively abnormal hemoglobin in β thalassemia, so diagnosis depends on quantitative hemoglobin electrophoresis assays for measurement of HbA, HbF, and HbA2 levels.

- In **β thalassemia major** there is no HbA because there is a total absence of β globin chain production. All of the hemoglobin is fetal, plus a small amount of HbA2.
- **β thalassemia intermedia** patients have HbF levels of 10-90%. There is a small amount of HbA2 and the rest is HbA.
- In **β thalassemia minor** there is a slightly elevated hemoglobin A2 level. This is partially a consequence of decreased hemoglobin A production so that the proportion of hemoglobin A2 is higher, and partly due to an increase in δ chain production in a rather feeble effort to compensate for the paucity of β chains. There is a small elevation in fetal hemoglobin as well. However, the majority of Hb is normal hemoglobin A.

B. α Thalassemia Syndromes

1. Genetics:

In α thalassemia there is a decrease in α globin chain production with normal β globin production. The α globin genes are tandemly duplicated on chromosome 16 (**fig 5.1**) such that the genes and flanking sequences are highly homologous. The high homology predisposes the α -globin gene cluster to unequal crossover events, deletions, and reduplications. In fact, deletion of one or both α globin genes on chromosome 16 is the most common mutation in the human genome. The highest incidence is in Africans and their descendents. In Southeast Asia, the prevalence reaches close to 40%.

Whereas β globin chains are present only in HbA and reach significant levels at about 6 months of age, α globin chains are a constituent of HbA, HbF and HbA₂. Thus α chain gene mutations affect all hemoglobins, and clinical manifestations are apparent at birth or even *in utero*. Because there are four α globin genes, there are four potential α thalassemia syndromes. In East Asian populations, both α genes are commonly deleted from the same chromosome. Thus, the combination of two of these chromosome defects would cause fetal death. In contrast, it is rare for both α genes to be deleted from the same chromosome in people of Mediterranean and African descent. It is more common in these populations for only one α gene to be deleted from each chromosome. As a result, the incidence of α thalassemia trait (α -/ α -) and the silent carrier trait ($\alpha\alpha$ / α -) is significant in these groups, whereas the incidence of hydrops fetalis (--/--) and hemoglobin H (--/ α -) disease is negligible. In Southeast Asia, both types of deletions are common, so that individuals with α thalassemia trait may have either an α -/ α - or a --/ $\alpha\alpha$ genotype. Only those with the latter genotype are at risk for having a severely affected infant.

2. Clinical features

As with β thalassemia, there is great genetic heterogeneity with many different defects of the α globin genes that lead to a variety of phenotypes. The typical clinical presentations are described below.

○ **Alpha Thalassemia (Silent carrier)**

When one α chain gene is thalassemic ($\alpha\alpha$ / α -), the affected individual is not sick and diagnosis is usually inferred from a family study. In the cord blood of neonates, there are increased γ chains forming Bart's hemoglobin (γ 4), which disappears after six months of life.

○ **Alpha Thalassemia Minor**

When two α chains genes are thalassemic (α -/ α - or --/ $\alpha\alpha$), there is microcytosis, hypochromia, mild anisocytosis, and poikilocytosis. Anemia is mild or absent. Bart's hemoglobin (γ 4) is increased in the newborn.

○ **Hemoglobin H disease**

When three α chain genes are thalassemic (α -/--), there is a chronic hemolytic anemia with a hemoglobin level usually about 8 to 10 gm/dl. In the newborn, 20% to 40% of hemoglobin is Bart's. This is replaced in older children and

adults by hemoglobin H (β_4), which varies between 5% and 30%. β_4 (HbH) inclusion bodies are found in red cells. These cells are rapidly removed from the circulation by the RE system. Microcytosis, hypochromia, target cells, anisocytosis, and poikilocytosis are seen in all clinically significant thalassemia syndromes. Anemia may become more severe after ingestion of oxidant drugs that accelerate oxidation and precipitation of hemoglobin H. The diagnosis is confirmed by hemoglobin electrophoresis.

- **Hydrops fetalis**

When four α chain genes are thalassemic (--/--), the affected fetus is usually premature and stillborn, or dies at birth. Hemoglobin electrophoresis shows mostly hemoglobin Bart's (γ_4). Hemoglobin Bart's, like Hemoglobin H, has a very high oxygen affinity and cannot deliver oxygen to the tissues. Death is caused by liver failure (the liver becomes engorged with nucleated red cells due to extensive extramedullary hematopoiesis) and tissue hypoxia.

3. Prevention and treatment of thalassemia

Screening couples at risk is performed by assessing the maternal MCV and ferritin. If the mother is found to have microcytosis with a normal ferritin, she and her partner are then evaluated for thalassemia. This approach to screening has been very effective worldwide.

The treatment of the severely affected thalassemia patient is mainly supportive. Regular transfusions are given to reduce the effects of the anemia on bone structure, spleen size, and growth and development. Although general health is much improved with regular transfusion, the resultant accumulation of iron had been a major threat to survival. Successful programs to remove excess iron from the tissues are now in widespread use. Patients are treated with continuous subcutaneous infusions of the iron chelator deferoxamine, which facilitates urinary excretion of iron and greatly prolongs life (**Fig. 5.7**). Oral iron chelators have recently come on the market and may eventually become the treatment of choice for many patients.

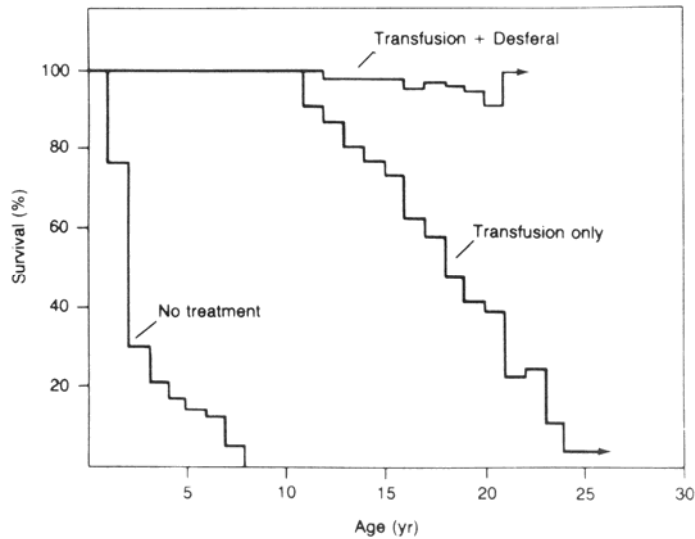


Fig 5.7 Thalassemia Major: Improved life expectancy with transfusion and iron chelation (Desferal = deferoxamine). Cao et al Cooley Foundation

- **Stem cell transplant**

Allogeneic bone marrow transplant is the only cure for thalassemia major. For those children with no hepatic toxicity from iron overload at the time of transplant, we see a 95% 3-year event-free survival rate after transplant. For older patients, the survival rate is about 80%.

V. Summary

The genetic diseases of hemoglobin became prevalent because they protect against malaria. Genetic counseling has markedly reduced the incidence of clinically significant thalassemia syndromes in much of the world. Therapies targeting the various factors that contribute to sickling (NO depletion, vasoconstriction, inflammation, cell-cell adhesion, cellular dehydration, and low Hb F) appear promising.

CHAPTER 6

BLOOD TRANSFUSION

Key Concepts:

- Principles of safe and effective blood component transfusion
- Biology and clinical significance of blood groups
- Clinical and laboratory features of transfusion reactions and other complications of transfusion

Learning Objectives:

1. Identify the various components of whole blood that can be transfused and list indications for transfusion of each component.
2. Define what is meant by a blood group and describe the biochemistry, genetics, and clinical significance of the ABO and Rh systems.
3. Describe the process of typing and crossmatching red cells for transfusion, and the method used to screen for unexpected red cell antibodies; explain the importance of each step.
4. Be able to recognize the various types of transfusion reaction using clinical and laboratory data, and describe the pathophysiology of each type of reaction.
5. List the major infectious complications of transfusion in order of frequency; describe the precautions taken to prevent these complications.

I. Introduction

A donation of blood is a tithe of one's vital essence—10% of the life's blood to some anonymous sick person who can not pay it back. It is a conspicuous demonstration of altruism and human goodness. It is also the basis of a huge industry. 14 million pints (units) of blood are processed each year from 8-10 million donors in this country to meet patient needs. Similar programs are carried out in most Western countries. However, blood supply and safety are uncertain in many developing nations. Although the blood itself is free, the costs of processing and administering it may result in a bill to the patient of up to \$600 per unit! Much of this chapter is devoted to explaining the processing and testing of blood as well as detailing the risks of its use.

FYI: History

A brief history of blood banking is instructive.

- 1628 Harvey discovered the circulation of blood.
- 1665 Shortly thereafter, Lower, in England, tried the first transfusions in modern times. He kept dogs alive by transfusion from other dogs.
- 1667 Denis in France and Lower in England transfused blood from lambs to humans. The next year, animal-to-human transfusion was outlawed, and advances were delayed for 150 years.
- 1818 Blundell in England performed the first direct transfusion for postpartum hemorrhage.
- 1900 Landsteiner of Austria discovered the ABO blood group.
- 1915 Lewisohn in New York City used citrate to anticoagulate blood, making storage possible.
- 1930 The first blood bank was established in England
- 1937 The first blood bank in this country was started at Cook County Hospital in Chicago.
- 1940 The Rh blood group was discovered.
- 1941 The American Red Cross established a national blood program.
- 1952 The plastic bag was introduced, replacing glass bottles.
- 1961 Platelet transfusion was recognized to stop bleeding in aplastic anemia and acute leukemia.
- 1962 Antihemophilic factor was commercially fractionated.
- 1967 Rh immune globulin was introduced to prevent immunization of pregnant women.
- 1971 Hepatitis B testing began.
- 1972 Apheresis was used to extract single components from blood, returning the remainder to the donor.
- 1985 HIV was recognized as a new hazard of blood transfusion, and testing was introduced.
- 1988 The etiologic agent for hepatitis C was identified, and testing was introduced. Subsequently more sophisticated tests for hepatitis C and HIV, and testing for HTLV-I and II and West Nile virus have been implemented.

-Am. Assoc. Blood Banks

II. Blood Components – see Table 6.1

The purpose of transfusion is to replace missing components of blood without undue risk to the patient. In current practice, whole blood is rarely given. The blood is fractionated in the blood bank laboratory into separate components. Some components, like plasma, albumin, and clotting factor VIII can be frozen or lyophilized and kept indefinitely. Others, like platelets, must be used within a few days. The most commonly transfused component is the red cell, followed by platelets, plasma, immunoglobulins, and clotting factor VIII (although recombinant clotting factor preparations have largely replaced plasma-derived concentrate). White blood cells are rarely transfused. A unit of transfused material is the amount retrieved from a single donation.

Thus a unit of red cells, platelets, white cells, or plasma is the amount recovered from a donated "pint" (450 ml) of blood.

When blood is donated, a plastic bag containing 65 ml of citrate/dextrose/phosphate/adenine (CPDA) mixture is filled to 500 cc. The blood is centrifuged, and the plastic bag is squeezed to force the plasma and platelets into a second bag, leaving behind packed red cells. The bag with plasma and platelets is then centrifuged, and the plasma is squeezed into a third bag, thus yielding plasma and a platelet concentrate. If all of the components of whole blood are required for replacement of massive hemorrhage, red cells, plasma, and platelets would be given as separate components.

Red cells survive up to 35 days at 4 °C in CDPA solution.

- Citrate prevents clotting;
- Dextrose is used for glycolysis;
- Phosphate and adenine help replenish ATP.

In spite of these nutrients, potassium is lost from red cells, the 2,3DPG concentration declines, and lactate accumulates (the storage "lesion"). The biochemical abnormalities are corrected after a day in the patient's circulation. Red cells in storage are lost at the rate of 1% per day as they are in the body, so that at 35 days, only 70% of the red cells are intact. **On average, a unit of packed red cells will raise the hemoglobin of an adult by 1 gram/dl.**

A. Red Cells

The primary indication for red cell transfusion is to establish adequate oxygen carrying capacity. Red cell transfusions are most commonly given to treat symptomatic anemia, hemorrhage, or surgical blood loss. The hazards of receiving blood include iron accumulation, antigenic stimulation, allergic reactions, fluid overload, and risk of infection. These risks will be taken up separately further in the chapter.

B. Platelets

The second most important component of blood (at least from a transfusion perspective) is the platelet. Each unit of blood yields about 5×10^{10} platelets. Platelet concentrates can be stored at room temperature for up to five days without loss of survival. **The primary indication for platelet transfusion is to establish adequate numbers of platelets to stop bleeding, prevent spontaneous hemorrhage, or prevent excessive bleeding during invasive procedures in patients who have low platelet counts or dysfunctional platelets.** Thrombocytopenia severe enough to require platelet transfusion is commonly encountered in patients with hematologic or other malignancies who undergo chemotherapy. A single unit of platelets transfused will normally raise the platelet count 6,000-8,000/ μ l.

A special problem for the patient who must receive platelets over weeks to months is the risk of developing antibodies that will destroy the platelets and make the transfusion ineffective. These antibodies may be directed against HLA antigens or other antigens on the platelet surface. In the past up about 40% of patients who received platelets repeatedly developed platelet refractoriness. This figure has declined significantly in recent years because the routine removal of contaminating leukocytes from platelet concentrates has reduced exposure to HLA antigens. Splenomegaly, fever, and sepsis also shorten survival of transfused platelets.

C. Fresh Frozen Plasma

The next most frequently used blood product is fresh frozen plasma (FFP). It is valuable because it contains all of the clotting factors. **The primary indication for transfusion of FFP is to achieve hemostasis by increasing the concentrations of circulating clotting factors** in patients with compromised liver function or other conditions that cause generalized clotting factor deficiency. FFP should not be given simply to expand blood volume.

D. Gamma Globulin

The next most commonly used plasma component is the immunoglobulin fraction, intravenous immunoglobulin (IVIG). IVIG confers passive immunity and is infused monthly to prevent bacterial infection in patients with poor antibody production. It also interferes with macrophage functions, and is used in a variety of autoimmune disorders such as autoimmune thrombocytopenia (ITP – see chapter 12).

Table 6.1 – Blood components that can be transfused*

| Component | Composition | Indication(s) |
|---------------------------|---|---|
| Red cells (packed) | RBC; trace WBC, platelets, plasma | Increase oxygen carrying capacity of blood |
| Platelets | Platelets, plasma, some RBC and WBC | Treat or prevent bleeding due to thrombocytopenia or dysfunctional platelets |
| Fresh Frozen Plasma (FFP) | Plasma, with all coagulation factors, complement | Treat or prevent bleeding due to multi-factor coagulopathy, severe liver disease |
| Cryoprecipitate | Fibrinogen, von Willebrand factor, factor VIII, factor XIII | Treat or prevent bleeding due to hypofibrinogenemia, factor XIII deficiency, von Willebrand disease. Fibrin glue for surgery. |
| Gamma globulin (IVIG) | IgG | Immunodeficiency disorders; some autoimmune diseases |

*Note: White blood cell (granulocyte) transfusions are sometimes given to granulocytopenic patients with severe infection, but this is not routinely done because the transfused cells have a very short half-life that limits the efficacy of such transfusions.

III. Blood Groups

The science of blood banking is built around the immunology of blood antigens and the system of procuring and fractionating the blood into its components. Most of this chapter, therefore, is devoted to understanding the immunology of the red cell.

Blood types are a reflection of genetic differences in cell membrane structure. These structural differences are important in clinical medicine because they can induce antibodies that may cause clinical disease; two examples are hemolytic transfusion reactions and hemolytic disease of the fetus and newborn. **A blood group system is a family of related antigens determined by alleles at a gene locus, or a cluster of linked genetic loci.** There are more than 20 systems of red cell antigens. The systems vary considerably in clinical importance. One of the goals of this chapter is to describe those red cell antigens that are the most important in blood transfusions and to explain why the systems vary in significance.

IV. Red Cell Antigen Systems

A. The ABO System

The ABO blood cell antigen group of red cell antigens was discovered in a simple experiment in 1901 by Karl Landsteiner, who took blood samples from himself and five of his colleagues, separated the serum from the red cells, and mixed each serum with a sample of each of the six red cells. In some of these serum-cell mixtures, he noted visible agglutination or aggregation of the red cells; in others there was no change in the red cell suspension. From the patterns of agglutination, Landsteiner proposed the ABO and blood cell antigen system.

1. Genetics and biochemistry

It was proven by 1910 that a person's ABO group is inherited. The ABO locus is on chromosome 9. The A and B genes are responsible for the production of different antigens. A weak antigen (H) is found on group O cells, and in small amounts on red cells of individuals of blood types A, B, and AB. Table 6.2 gives the antigen and antibody complement in the four phenotypes and the possible genotypes for each phenotype.

The ABO antibodies are usually IgM, but may be IgA and/or IgG. The reason for sustained IgM production is not known. It is typical of such antibodies that they react well at room temperature or below ("cold-reacting") and do not need additional reagents to enhance agglutination ("saline-reacting"; "complete"). **Group O persons make both IgG and IgM anti-A and anti-B, whereas Group A and B individuals make mainly IgM anti-B and anti-A, respectively. Anti-A and anti-B antibodies are easy to detect in the laboratory. Regardless of their immunoglobulin type, they produce visible agglutination of A and B red cells, respectively.**

The ABH antigens have been demonstrated on almost all body cells and are important in organ transplantation as well as blood transfusion. 80% of normal people ("secretors") have ABH antigens in saliva, sweat, tears, urine, bile, milk, and other body secretions. The ability to form the water-soluble antigens requires an additional secretor gene.

Table 6.2

ABO PHENOTYPES, GENOTYPES, ANTIGENS AND ANTIBODIES

| <u>Phenotype</u> | <u>Frequency</u> | <u>Genotypes</u> | <u>Antigens on Red Cell</u> | <u>Antibody in Serum</u> |
|------------------|------------------|------------------|-----------------------------|--------------------------|
| O | 44% | O/O | H | Anti-A, -B, -AB |
| A | 42% | A/A A/O | A, small amount of H | Anti-B |
| B | 10% | B/B B/O | B, small amount of H | Anti-A |
| AB | 4% | A/B | A, B, small Amount of H | None |

In classical genetic terminology, **the O gene is recessive to the A and B genes** and the O gene phenotype can be detected only when the O gene is present in double dose—i.e., in group O. The A and B genes are both dominant over O. Thus, the heterozygotes, A/O and B/O, cannot be distinguished from the corresponding homozygotes, A/A and B/B, by typing of the red cells. **The A and B genes are codominant to each other**, so the A/B heterozygote has both A and B antigens on each cell.

The A, B, and H antigens are located on glycosphingolipid molecules. The carbohydrate chains are synthesized by the sequential action of transferase enzymes. Each transferase adds a specific sugar to a substrate. The H chain ends with a terminal fucose, a required substrate for enzymes produced by the A and B genes. A, B, and O are alleles of the same gene encoding a sugar transferase. Enzymes encoded by different alleles differ in several amino acids critical for enzyme specificity. The A gene produces an enzyme that adds N-acetyl-galactosamine to the H chain. The product is called an A chain. The B gene produces an enzyme that adds the galactose, converting H chains to B chains. The O gene is allelic to the A and B genes but encodes a truncated protein without transferase activity. Group O chains are unaltered H chains (Fig. 6.1).

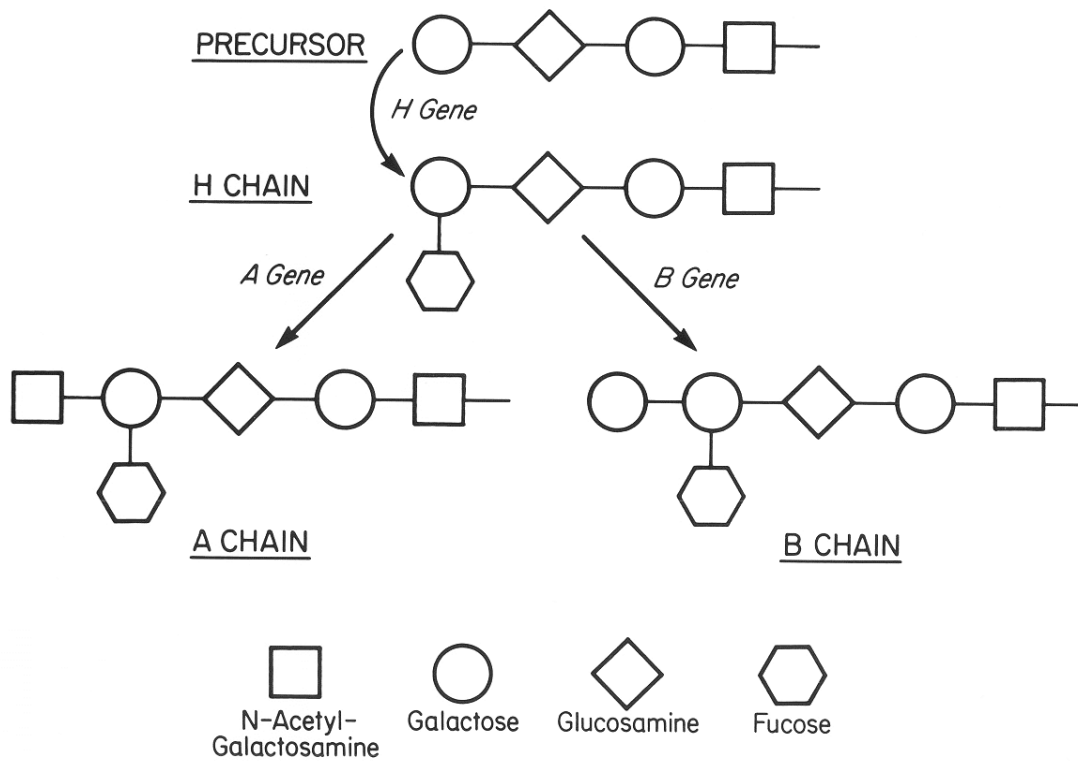


Figure. 6.1. Biochemistry of the ABO blood group. The H gene adds a fucose to the end of the glycosphingolipid chain. Under A gene influence an N-acetyl-galactosamine is added. The B gene adds a galactose at the same site. The O gene codes for a non-functional protein, and neither sugar is added; the H chain is therefore unmodified.

Table 6.3 Frequency of ABO phenotypes in various populations

| Phenotype | African-American | Asian | Caucasian | Hispanic |
|-----------|------------------|-------|-----------|----------|
| A | 27% | 27 | 40 | 28 |
| B | 20 | 25 | 10 | 12 |
| AB | 4 | 5 | 5 | 4 |
| O | 49 | 43 | 45 | 56 |

2. Antibodies

Anti-A and anti-B antibodies are called "naturally occurring" and "regularly occurring," because they are always found in the blood of normal persons who lack the corresponding antigen without prior immunization. The carbohydrate chains carrying the ABH antigens are present on cell membranes of the fetus and newborn, but the child does not begin to synthesize A and B antibodies until three to six months of age. Once antibody production begins, it continues for life (although antibody titers tend to decline in old age).

Why do these antibodies appear? The current view is that they are a product of an immune response to similar antigens in the environment. Carbohydrate chains similar to the A, B, and H chains are found on the cell membranes of bacteria, plants, and other animal species. An infant comes in contact with these carbohydrate chains in food or via the bacterial flora of the gastrointestinal tract. Those chains that are not present on an infant's own cells will induce an antibody response. For example, the group O infant will make antibodies against both the A and B antigens. The group A child will make only anti-B, and the group B child, anti-A. Why are no anti-H (anti-"O") antibodies formed? The enzymatic conversion of H to A to B chains is not total. A few uncovered H chains remain on the cells of A, B, and AB individuals, presumably rendering them tolerant to H chains.

3. Clinical importance

The ABO system is the most important of all blood group systems because of the naturally and regularly occurring antibodies. Anti-A and/or anti-B antibodies are found in 95% of normal persons—all but those who are of group AB. **If the ABO groups of patient and donor are not matched when blood is selected for transfusion, the patient's anti-A and/or anti-B antibodies may destroy the transfused red cells, sometimes leading to the death of the patient.** The risk of potentially lethal ABO mismatch from a random red cell transfusion would be about 30%.

Therefore, the first step in pretransfusion testing is to determine the patient's ABO group. Donor blood of the same ABO group is selected for transfusion. Such blood is said to be "ABO compatible" and will not be attacked by naturally and regularly occurring antibodies in the patient's plasma. In an emergency when there is insufficient time to type the patient's blood, we may transfuse group O red cells. We remove the plasma from the donor blood and give only the red cells, since the red cells of a recipient who is not group O may be damaged by anti-A or anti-B antibodies in the donor plasma.

B. The Rh System

From 1900 to 1940, blood transfusions were sometimes followed by hemolysis of the donor blood even though the patient and donor were of the same ABO group. No unusual red cell antibodies could be found in the sera of patients who had suffered such reactions. In 1939, Levine and Stetson discovered an antibody that was related to clinical transfusion reactions. The patient was a woman who had just given birth to a stillborn infant that had died from hemolytic anemia (hydrops fetalis). This was her second pregnancy. Because of bleeding during delivery, she was transfused with blood from her husband. A severe hemolytic reaction occurred in spite of the fact that she and her husband were of the same ABO group.

When Levine retested the woman's serum, he found an antibody that reacted with her husband's red cells as well as the cells of 80 of 104 ABO-compatible donors.

He postulated that the woman had been immunized to this new blood group antigen by her baby through the following sequence of events:

- The fetus inherited the antigen from the father.
- A few fetal red cells crossed the placenta into the mother's circulation.
- The mother made antibody against the foreign antigen.
- The antibody crossed the placenta and attacked the fetus' red cells, producing a severe hemolytic anemia.
- The fetus died from severe hemolytic anemia.

After delivery, when the woman was transfused with her husband's blood, this same antibody destroyed the transfused red cells, causing a second "disease," a hemolytic transfusion reaction.

Anti-Rh antibodies were found in the sera of some patients who had had hemolytic transfusion reactions, and in the sera of some mothers of babies with hemolytic disease of the newborn. Rh antibodies were probably present in more of these patients, but were too weak to be detected in the laboratory. **Rh antibodies are usually IgG**, and, unlike the ABO antibodies, they do not readily agglutinate red cells suspended in saline solution. During the next few years, four other antibodies and their corresponding antigens were identified and categorized as part of the Rh antigen group: C, E, c, and e. When methods were developed to detect IgG antibodies, many other rare Rh antigens were discovered.

1. Genetics and biochemistry

The Rh genes are located on the short arm of chromosome 1. There are two closely linked genes, one for the D protein, the other for Cc/Ee. An individual who is Rh(D) negative may have a homozygous deletion of the D gene or an active suppressor gene. In rare individuals, both the D and the Cc/Ee genes are nonfunctional (Rh null) and a chronic hemolytic anemia results. This suggests that the Rh proteins serve a function on the red cell membrane other than causing trouble for the blood bank.

The Rh system is the most complex of the human blood group systems. There are now more than thirty antigens. Rarities such as weak or absent antigens also occur. Table 5.4 lists the more common antigens and genotypes. **Routine pretransfusion testing only tests for the original Rh antigen, Rh(D). The terms "Rh-positive" and "Rh-negative" refer only to the presence or absence of the immune-dominant Rh(D) antigen.** Approximately 85% of whites are Rh-positive and 15% are Rh-negative. Among African-Americans, about 92% are Rh-positive and 8% are Rh-negative.

Table 6.4
THE MORE COMMON Rh GENOTYPES

| <u>Genotype</u> | <u>Frequency</u> <u>Wis. Whites</u> | <u>Rh</u> <u>"Type"</u> |
|-----------------|--|----------------------------|
| 1. DCe/dce | 32% | + |
| 2. DCe/DCe | 7% | + |
| 3. dce/dce | 16% | - |
| 4. DCe/DcE | 14% | + |
| 5. DcE/dce | 13% | + |
| 6. DcE/DcE | 2% | + |

2. Antibodies

Antibodies against the Rh antigens are uncommon ("irregular"). This is the opposite of the situation in the ABO system. Anti-Rh(D) antibodies appear in fewer than 5% of Rh(D) negative persons. Antibodies against the C and E antigens are even more uncommon. **When Rh antibodies are found, they are almost always the result of immunization by previous blood transfusions or by pregnancy.**

The Rh(D) antigen is highly immunogenic. Approximately 50% of Rh(D)-negative persons will be immunized by a single exposure to the antigen through red cell transfusion (Table 6.5). Another 20% will make antibody after a second antigenic stimulus. The remaining 30%, called "non-responders," cannot be immunized. Rh antibody appears in the serum two to six weeks after antigenic stimulation. Often the first antibody to be detected is IgG ("warm-reacting"). Because the antibody is IgG, it is also "incomplete", i.e., it needs special methods for detection. Significant amounts of IgM antibody are found in only a minority of patients.

The Rh(D) antigen is at least twenty times more effective as an antigenic stimulus than the other Rh antigens (Table 6.5).

To summarize:

- **The D antigen is the most important in the Rh system**
- **Other antigens in this system include C,c,E,e**
- **Rh positive (DD or Dd) = 85% of people**
- **Rh negative (dd) = 15% of people**
- **Anti-D antibodies are NOT naturally occurring in Rh-negative people**
- **Anti-D antibodies develop only after exposure to the Rh antigen, e.g., via transfusion or pregnancy**

Table 6.5
RELATIVE ANTIGENIC POTENCY

| <u>Antigen</u> | <u>Potency*</u> |
|----------------|-----------------|
| D (Rh) | 50% to 70% |
| Kell | 10% |
| c | 3% |
| E | 3% |
| Duffy | 0.4% to 1% |
| Kidd | < 0.2% |

*Percentage of persons who lack the antigen who would produce antibody after one exposure to the antigen (one transfusion).

3. Clinical importance

In all blood group systems, clinical importance is directly proportional to the incidence of blood group antibodies. **Because an Rh(D)-negative person is very likely to be immunized by a transfusion of Rh(D)-positive blood, the second step in pretransfusion testing is to determine the patient's Rh(D) antigen type (Rh positive or negative).** Testing for other Rh antigens such as C/c and E/e is not routinely done. Once the Rh type is known, then donor blood of this Rh type is selected. Such blood is called "type specific." This means that the ABO group and Rh type of the patient and donor are the same.

In summary, ABO matching is done to prevent an immediate hemolytic transfusion reaction. If the patient and donor Rh types are different, there usually are no immediate hemolytic consequences. Rh matching of patient and donor is done to prevent such immunization, not to prevent a hemolytic transfusion reaction. The rationale for preventing immunization applies primarily to women in the child-bearing age: an Rh-negative woman who has Rh antibodies may be unable to bear children because, if the child is Rh-positive, it may die *in utero* from hemolytic disease of the newborn.

Hemolytic transfusion reactions due to anti-D occur infrequently because of the standard practice of transfusing only Rh-negative blood to Rh-negative patients. Clinical problems due to antibodies against the various C and E antigens are even less common because these antigens are only weakly immunogenic. When problems do occur, they take the form of hemolytic disease of the newborn or hemolytic transfusion reactions.

C. Other Red Cell Antigen Systems

1. Antigens

Some of the other known systems of red cell antigens are listed in Table 6.6. The M, N, and S antigens were discovered through deliberate experiments in which animals were immunized with human red cells. The others were almost all discovered as a consequence of clinical problems with transfusion reactions or hemolytic disease of the newborn.

Table 6.6

OTHER RED CELL ANTIGEN SYSTEMS

| <u>System</u> | <u>Major Antigens</u> | <u>Comments</u> |
|---------------|-----------------------|--|
| Kell | K(ell), k(Cellano) | Anti-K (Kell) is the most commonly occurring red cell antibody after anti-Rh(D). Among whites, 10% are Kell-positive, and 90% Kell- negative. |
| Duffy | Fy-a, Fy-b, Fy-- | Null gene (Fy--) very common in blacks (70%). Fy-- (null) has greater resistance to malaria. |
| Kidd | Jk-a, Jk-b | Antibodies to Jk-a may cause delayed hemolytic reaction. |
| MNSs | M, N, S, s | Occasionally we find spontaneously occurring antibodies to M.* |
| Lewis | Le-a, Le-b | Antigen located on same chain as ABO antigens. Antibodies usually IgM.* |
| I | I, i | Antigens located on same chain as ABO. The common "cold agglutinin" found in cold agglutinin disease is an anti-I with increased thermal amplitude.* |

*IgM antibodies occur sporadically. They usually react with red cells only at temperatures below 37 °C, so do not cause clinical disease but cause problems in laboratory testing.

2. Genetics and biochemistry

In most of the systems, there are two codominant alleles. In the last several years, researchers have begun to decipher the structures and genetic basis of these "minor" red cell antigens.

3. Antibodies

Antibodies against these "minor" red cell antigens are very uncommon. They are formed as a result of direct immunization by blood transfusion or, in women, pregnancy.

4. Clinical aspects

In the course of routine pretransfusion testing, no attempt is made to determine the patient's complete Rh phenotype or Kell, Duffy, Kidd, or MNSs type, and no attempt is made to match patient and donor for their complements of these various antigens. We know that a transfusion of red cells will expose the patients to antigens that are foreign to them. The risk of immunization is small due to the weak antigenicity of these antigens (Table 6.5). "Unexpected" blood group antibodies—i.e., antibodies other than anti-A and anti-B—are found in only 1% to 2% of all patients, and many of these are anti-Rh(D) (Table 6.7). When transfusions must be given to one of these immunized patients, the antibody must be detected and identified, and donor blood selected that lacks the specific antigen. In other words, **to prevent a hemolytic transfusion reaction in a patient with an "unexpected" blood group antibody, the donor red cells should lack the antigen corresponding to the specificity of the antibody present in the patient's serum. Antibody screening and (when appropriate) cross matching accomplish this.**

To summarize:

- Hemolysis occurs only when the antigen (on the donor red cells) and the antibody (in the recipient's plasma) are in the same place (the recipient's bloodstream) at the same time.
- If the recipient has an antibody to a particular red cell antigen but the transfused blood lacks that specific antigen, the transfusion will be safe.

Table 6.7

INCIDENCE OF UNEXPECTED* BLOOD GROUP ANTIBODIES

| | |
|------------------------|-----------------------------|
| Anti-D (Rh) | 0.5% (= 3% of Rh-negatives) |
| Other Rh (E, c, e, Ce) | 0.5% |
| Kell | 0.3% |
| Fy-a | 0.1% |
| All Others | 0.1% |
| TOTAL | 1.5% of all patients |

- * Unexpected in the sense that they are found only occasionally in those people who lack the antigen, people who have been transfused, or in multiparous women. The antibodies are usually IgG immunoglobulins and are detected only by using special laboratory methods, including Coombs (antiglobulin) test.

FYI: Other Blood Group Antigens

HLA

The HLA antigens are transmembrane glycoproteins involved in self-recognition. Class I antigens (HLA-A,B,C) are found on all nucleated cells except spermatozoa, and the trophoblast. Red cells carry insignificant amounts of HLA antigen but platelets probably adsorb HLA antigen from the plasma. Class I antigens have some homology with immunoglobulin molecules. They are recognized by cytotoxic T cells, and are the primary targets in graft rejection.

Class II antigens (HLA-DP,DQ,DR) are generated by immune response genes. They are found only on hematopoietic progenitors, endothelial cells, monocytes, macrophages, B cells, and some T cells. They are involved in antigen processing and the generation of effector T cells. Structurally, the Class II antigens also have some homology with immunoglobulin molecules. Class I and II antigens are inherited as haplotypes, half from each parent, so that one in four siblings is likely to be identical. They are extremely diverse: more than 40 antigens have been defined in the HLA-B region alone. HLA antigens are very important in organ transplantation. Blood and platelet transfusions may result in immunization to HLA antigens, mainly from contaminating leukocytes. These antibodies can jeopardize the success of organ transplants or platelet transfusions.

Granulocyte antigens

Several unique granulocyte antigens have been described (NA1, NA2, etc.). Antibodies against these antigens occasionally may cause neonatal granulocytopenia secondary to maternal antibodies, a syndrome analogous to hemolytic anemia of the fetus and newborn. They also cause autoimmune neutropenia. Febrile reactions and pulmonary infiltrates after transfusion are usually due to antibodies against leukocytes. Tests for these antibodies are not generally available. The febrile reactions can often be reduced or avoided by filtering blood to remove leukocytes.

Platelet antigens

Four systems of platelet-specific antigens are known: Pl^A , Pl^E , Ko , and $DUZO$. Antibodies against one of these antigens may cause neonatal alloimmune thrombocytopenia due to antibody produced by the mother in a manner analogous to hemolytic disease of the newborn (see chapter 7). A rare syndrome of post-transfusion purpura may occur in association with platelet-specific antibodies, usually anti- Pl^A . Antibodies against HLA antigens shorten the lifespan of transfused platelets so that some chronically transfused patients no longer benefit from random donor platelets. In this situation, HLA-matched platelets are given, usually with good results.

Antigens on plasma proteins

Variations in the structure of complement proteins and immunoglobulins, both IgG and IgA, occur. Antibodies against IgA in transfused plasma may cause serious transfusion reactions, including urticaria, wheezing, and rarely anaphylaxis especially in IgA deficient patients.

IV. Standard Pretransfusion Testing

A. The Tests

The tests that are ordinarily done prior to elective transfusion of red cells are listed in Table 6.8.

1. ABO typing and matching

ABO typing and matching of patient and donor ABO groups is the first and most important step. If this were not done, the donor and/or patient red cells would be destroyed by the normally occurring anti-A and anti-B antibodies.

2. Typing for the Rh(D) antigen

Typing for the Rh(D) antigen and transfusion of Rh-negative donor blood to Rh-negative patients is standard practice. The Coombs test (see below) is used to screen for the RhD antigen.

Table 6.8

STANDARD PRETRANSFUSION TESTS FOR NONEMERGENCY TRANSFUSIONS OF RED CELLS AND WHOLE BLOOD

On Donor Blood

1. ABO group
2. Rh(D) type (positive or negative); Rh-negatives also tested for weak D (Du).
3. Serum tested for unexpected antibodies to red cells.
4. Tests for infectious agents and evidence of infection.
 - Hepatitis B (antibodies to core antigens, presence of surface antigen in serum)
 - Hepatitis C (antibodies, nucleic acids)
 - Hepatitis—general (serum transaminase level)
 - HIV-1 (antibodies, nucleic acids)
 - HIV-2 (antibodies)
 - HTLV-1 (antibodies)
 - HTLV-2 (antibodies)
 - Syphilis (antibodies)
 - West Nile virus (nucleic acids)

On Patient's Blood

1. ABO group.
2. Rh(D) type (positive or negative, no test for Du).
3. Select donor blood of same ABO/Rh as patient ("type- specific").
4. Antibody screening with patient serum vs. reagent red cells. This test is negative (no antibodies) in 99% of patients.
5. Cross match with patient serum vs. donor red cells. Blood usable only if cross match "compatible"—i.e., serum contains no antibody that can react with donor cells.

3. Antibody screening

Antibody screening identifies the 1% to 2% of patients who have an unusual, unexpected antibody but not the specificity of the antibody. A sample of the patient's serum is tested against a series of test red cells. These reagent cells are all group O and have been typed for about 30 antigens in the Rh, Duffy, Kidd etc. systems—the ones that are the most important in clinical practice—and a sufficient number of different reagents are used in the test to assure that each of the antigens is present on at least one of the test cells. If the patient's serum does not react with these cells, it is concluded that no "unexpected" antibodies are present, and the patient can be safely transfused with blood that is "type-specific," that is, of the correct ABO and Rh type, without regard for any of the other known red cell antigen systems. (But additional testing—the cross match—is done to insure that this judgment is correct. See below.) Because all the test cells are group O, the patient's regularly occurring ABO antibodies are not detected in the antibody screen; only "unexpected antibodies" are found.

In about 1% of patients, positive reactions are observed in the antibody screening. In such a patient, the next step is to test the serum against additional reagent red cells to identify the specificity of the antibody, a procedure called "antibody identification." If the offending antigen is very common, donor blood is then specifically typed, and donor units are selected that lack the antigen to which the patient has antibody.

4. Cross Matching

Cross matching—mixing a sample of the patient's serum with a sample of the donor red cells—is done as a double-check on the results of the ABO typing and antibody screening. To be sure the ABO typing of both patient and donor are correct, a sample of the patient's serum is spun with an aliquot of the saline-suspended donor red cells that are to be transfused. This is called an "immediate spin" and detects antibodies to ABO antigens. If no agglutination is seen, the donor and the patient are indeed properly ABO matched. **This step is done in all cases.**

Additionally, if the patient's antibody screen was positive, a full cross match is done with patient serum and the donor cells, including a Coombs test (see below). If agglutination is detected, the corresponding antigen is present on the red cells of this particular blood donor. This blood is "incompatible" for this patient, and must not be transfused. The cross match is repeated with additional donor units until a sufficient number that do not react with the patient's antibody are found. If the donor red cells are not agglutinated by the patient's serum, they are said to be "compatible," meaning that post-transfusion survival of these cells probably will not be shortened by antibody in the patient's plasma. **The Coombs test portion of the cross match is not routinely performed if the antibody screen is negative.**

B. Methods Used in the Antibody Screening and Cross Match Procedures

1. The phenomenon of agglutination

All routine test methods used today depend on agglutination reactions. Antibody is mixed with red cells in the test tube, and a positive reaction is indicated by agglutination of the test cells. All antibody molecules have at least two combining sites. Agglutination is the visible consequence of the attachment of the combining sites on one antibody molecule to two different red cells, forming a protein bridge that binds them together. When sufficient antibody cross-linkages form, agglutination can be observed with the naked eye. But agglutination will occur only if the cells can approach each other closely enough for one antibody molecule to attach to two different red cells. We have little control over the distance between combining sites on an immunoglobulin molecule—this is an intrinsic property of the molecule. The laboratory manipulations then consist of changing the distance between red cells.

Red cells repel each other because of negatively charged amino acid side groups and ionized sialic acid groups on the membrane. The distance between the red cells is, in part, determined by the ability of the suspending medium to dissipate this electrical repulsion, the zeta potential or dielectric constant.

With red cells suspended in 0.85% saline, IgM can bridge the intercellular gap and produce visible agglutination (Fig. 6.2) at room temperature. IgG antibodies can bind to red cell membrane antigen, but a second red cell does not ordinarily approach closely enough for visible agglutination to occur (Fig. 6.3). This antibody reaction is called "incomplete".

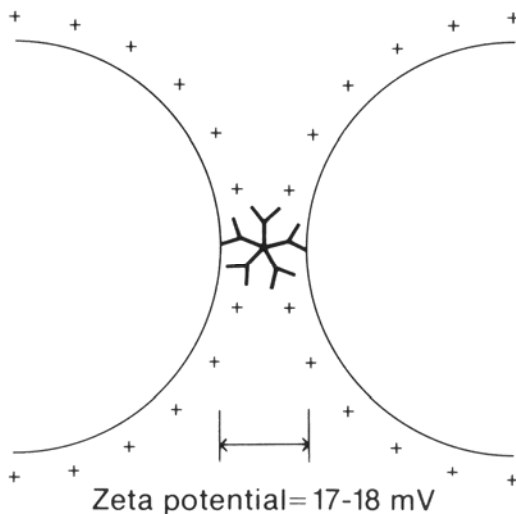


Figure 6.2. The large IgM pentamer is able to agglutinate saline-suspended red cells. The positive charges represent Na⁺ ions attracted to negatively charged sialic acid groups. (Redrawn from Ortho Diagnostic Systems, Inc., New Jersey. Bulletins: Ortho Broad Spectrum Anti-Human Serum (1970) and Ortho Bovine Albumin).

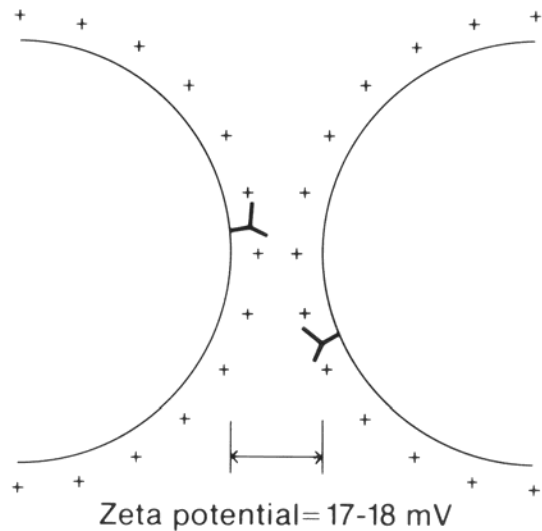


Figure 6.3. The effective length (distance between combining sites) of IgG molecule is too short to produce visible agglutination of saline-suspended red cells. (Redrawn from Ortho Diagnostic Systems, Inc., New Jersey. Bulletins: Ortho Broad Spectrum Anti-Human Serum (1970) and Ortho Bovine Albumin).

Several methods are used to reduce the intercell distance and allow agglutination of red cells by IgG antibody:

- Suspend the cells in a medium with a greater charge-dissipating capacity such as bovine albumin or synthetic polymers such as dextran, PVP, or Ficoll.
- Modify the red cell membrane with sialidase, reducing the electrical charge and the water of hydration.
- **Link the antibody molecules with a second antibody** (Fig. 6.4, 6.5). This is the method routinely used in blood banking.

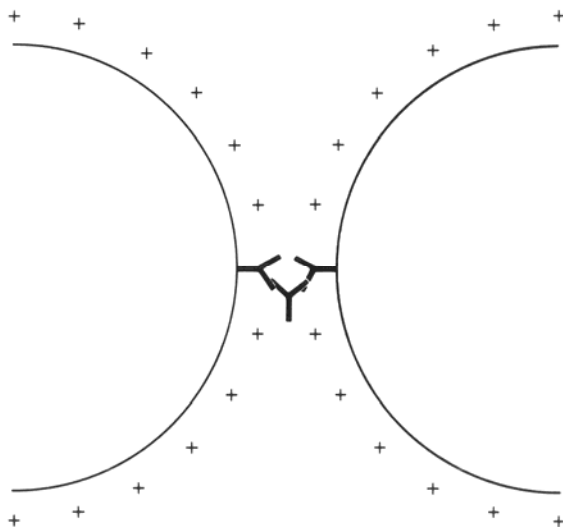


Figure 6.4. The Coombs or antiglobulin reaction. The IgG molecules that have bound to red cell membrane antigen are linked by adding a rabbit antibody, which attaches to the heavy chains of the human immunoglobulin. The red cells are now cross-linked by a three-way immunoglobulin bridge. (Redrawn from Ortho Diagnostic Systems, Inc., New Jersey. Bulletins: Ortho Broad Spectrum Anti-Human Serum and Ortho Bovine Albumin).

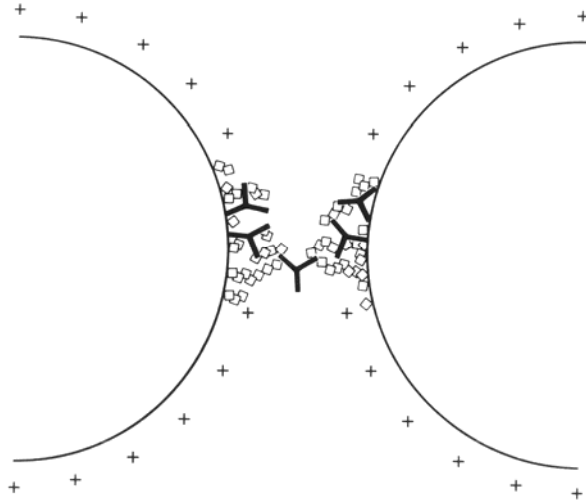


Figure 6.5. "Broad-spectrum" Coombs serum also contains antibody against complement proteins (C3 and C4), so it will cross-link red cells that have complement (open boxes) bound to their surfaces. (Redrawn from Ortho Diagnostic Systems, Inc., New Jersey. Bulletins: Ortho Broad Spectrum Anti-Human Serum and Ortho Bovine Albumin).

2. The Coombs or antiglobulin technique

The rationale of the Coombs technique is to link two antibodies or complement molecules together by an anti-antibody to bridge the distance between red cells and produce visible agglutination. A rabbit is immunized against human IgG and complement proteins. The serum from such an animal can be used as a reagent to detect IgG and/or complement bound to the surface of red cells. The technical name of this reagent is an "antiglobulin," that is, an antibody against immunoglobulin.

In the cross match and antibody screening procedures, donor or reagent red cells, respectively, are first allowed to react with patient serum at 37 °C to allow maximal binding of blood group antibody to red cell membrane antigen ("warm-reacting antibody"). The cells are washed with saline to remove unbound antibody. Then Coombs antiglobulin serum is added. If IgG from the patient serum has become attached to the donor or reagent red cells, the rabbit antibody will attach to it, forming a three-piece bridge between adjacent red cells (red cell antibody, rabbit antibody, red cell antibody). See Fig. 6.4. When used in this way, this test is often called the "**indirect Coombs test.**"

An antibody screening procedure must detect both IgM and IgG antibodies. The technique always includes a saline phase for IgM antibody and a Coombs (or antiglobulin) phase for IgG.

3. The direct Coombs test (direct antiglobulin test)

The Coombs technique can also be used to detect antibodies and/or complement bound to red cells in a patient's own circulation. Examples are:

- autoimmune hemolytic anemia, in which the patient makes antibodies to his or her own red cells;
- hemolytic disease of the newborn, in which antibody from the mother crosses the placenta and attacks the child's red cells;
- a hemolytic transfusion reaction, in which the donor red cells are attacked by antibody from the patient's plasma.

A blood sample is drawn, and the red cells are washed several times with saline to remove all nonattached immunoglobulins. Then the Coombs or antiglobulin reagent is added. If agglutination occurs, IgG and/or complement proteins are present on the patient's red cells. **This test is called the "direct" Coombs test or the direct antiglobulin test (DAT).**

To summarize:

- **The direct antiglobulin (direct Coombs) test detects antibodies bound to a patient's red cells. It is useful in the evaluation of hemolytic anemia.**
- **The indirect antiglobulin (indirect Coombs) test detects antibodies present in a patient's serum. It is used by the blood bank to detect antibodies that might cause hemolysis of transfused red cells.**

V. Transfusion Reactions

A. Infections

A variety of infections can be transmitted by blood transfusion. Those of greatest concern are hepatitis B and C viruses and HIV, because these viruses are relatively common and often lead to chronic infection with serious morbidity. Fortunately, the risk of infection with these viruses has diminished significantly in recent years thanks to improvements in donor screening and laboratory testing of donated blood. The risk of acquiring hepatitis B from a red cell transfusion is estimated to be around 1 per 150,000 units; the risk of acquiring hepatitis C or HIV is now less than 1 per million units. Other infections that can be transmitted by blood include cytomegalovirus, West Nile virus, human herpesvirus 8, HTLV-1&2, toxoplasma, malaria, Chagas disease, syphilis, and the human form of bovine spongiform encephalopathy (mad cow disease). A potentially lethal infection can develop from transfusion of bacterially contaminated red cells or platelets.

Traditionally, patients with chronic anemia were transfused when the Hgb fell below 10 gm/dl. The reason for choosing this threshold was the observation that the cardiac output begins to increase at hemoglobins below 10 gm/dl. The emergence of HIV and recognition of the potential viciousness of hepatitis C, however, forced the medical community to re-examine this criterion. The threshold for giving blood in cases of chronic anemia is now 7-8 gm/dl, unless there is an underlying medical condition that causes the patient to tolerate this degree of anemia poorly (e.g., heart or lung disease, old age). The risks of HIV and hepatitis C transmission have been diminished by screening, but more subtle problems such as immune impairment and increased risk of cancer are being described. Table 6.9 outlines some transfusion risks.

Table 6.9
RISKS OF RED BLOOD CELL TRANSFUSION

| Risk | Frequency per transfusion |
|----------------------------|---------------------------|
| Infection | |
| cytomegalovirus | common |
| Hepatitis B | 1/350,000 |
| HIV | 1/1,400,000 |
| Hepatitis C | 1/1,200,000 |
| Immune reactions | |
| fever, chills, urticaria | 1/50-1/100 |
| acute hemolysis | 1/76,000 |
| acute hemolysis with death | 1/1,800,000 |
| acute lung injury (TRALI) | 1/1300-1/5000 |
| delayed hemolytic rxn | 1/2500-1/10,000 |
| Graft vs. host phenomena | unknown but real |

B. Immune Transfusion Reactions

Immunologic reactions are due to interaction between antibody and antigen. Usually the antibody is present in the patient's plasma before the transfusion, and it reacts with some cellular or protein component of the donor blood. The offending antigen may be located on the cell membrane of donor red cells, platelets, or leukocytes, or on donor plasma protein molecules.

C. Immune Hemolysis

Two to five percent of patients experience a transfusion reaction when they receive blood transfusions. Transfusion reactions vary in severity from minor problems, such as one or two hives, to death. Of 70 fatalities associated with blood transfusion, 56% were due to hemolytic reactions and **half of those were due to clerical error in preparing or administering blood.** Fortunately such events are rare (Table 6.9). Studies by blood banks have shown that the tendency to human error is such that there always is a chance of mix-up, no matter how well-trained and well-meaning the personnel.

D. Intravascular Hemolysis

Intravascular hemolysis usually occurs because the patient is inadvertently given donor blood of the wrong ABO group. It is likely to occur whenever group A, B, or AB blood is given to a group O patient, group A or AB blood is given to a group B patient, or group B or AB blood is given to a group A patient. In any of these circumstances the donor red cells are attacked immediately in the patient's plasma by circulating antibodies, which are almost always complement-binding. Activation of the complement system produces holes in the membrane of the donor red cells, and the sequence of intravascular hemolysis described in Chapter 4.

ABO mismatched transfusions are accompanied by immediate and severe symptoms, usually beginning with shaking chills and fever. The patient may complain of headache or pain in the chest, back, or extremities. Shortness of breath (dyspnea), nausea, vomiting, and diarrhea are common. One of the most serious changes is **hypotension**, which may be severe enough to constitute outright **shock**. Hypotension is accompanied by compensatory tachycardia. Peripheral vasodilation results from the release of vasoactive compounds such as serotonin and histamine from platelets and mast cells, and complement fragments (C3a and C5a). Norepinephrine released from the adrenal medulla constricts the renal arterioles and causes renal cortical ischemia. Necrosis of the cells lining the renal proximal convoluted tubules may occur. The renal ischemia results in a period of **oliguria or anuria** of varying duration (Fig. 6.6)

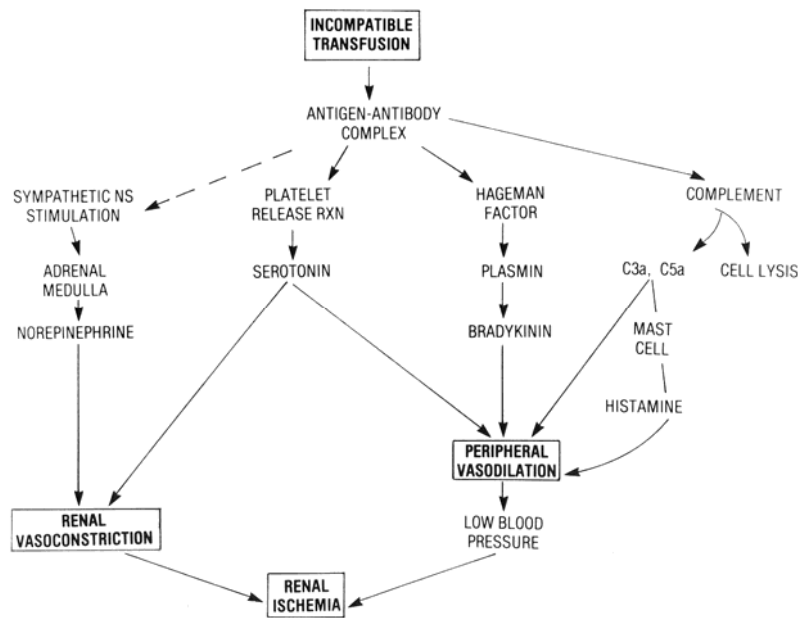


Figure 6.6. Pathophysiology of vascular complications of incompatible red cell transfusion. The kidney is the target organ. (Adapted from Goldfinger, D: Complications of Hemolytic Transfusion Reactions: Pathogenesis and Therapy in *New Approaches to Transfusion Reactions*, American Association of Blood Banks).

There is also a high likelihood of **disseminated intravascular coagulation (DIC)**. The clotting system is activated by a direct effect of antigen-antibody complexes and by subendothelial tissue factor exposed as a result of endothelial injury from activated complement and neutrophils, and is accelerated by phospholipids released from platelets, leukocytes, and the stroma of the lysed red cells. The syndrome of DIC is discussed further in chapter 12.

The adverse effects of hemolysis are dose-related: the more incompatible blood the patient receives, the worse the consequences. Review of the clinical course of adult patients who have received 200 to 500 ml of ABO-incompatible blood showed that 85% to 90% experienced an immediate, but self-limited, episode of shock and anuria lasting one to two hours. However,

some patients died of irreversible shock within a few hours. **The overall mortality from acute hemolytic transfusion reactions has been estimated at 10%.**

E. Immediate Extravascular Hemolysis

Extravascular removal of red cells from the circulation occurs when antibody is bound to red cell membrane antigen without complete activation of complement. The IgG-coated red cells are phagocytized by macrophages. **Extravascular hemolytic reactions are rarely fatal.** The immediate symptoms are usually limited to chills and fever. If phagocytosis is rapid, bilirubin excretion by the liver may not keep pace with production, and jaundice may begin after a few hours. Mismatches in the Rh, Duffy, Kell, or Kidd system usually fit this description.

F. Delayed Hemolysis

Since the patient and blood donor are ordinarily matched only for their ABO and Rh(D) type, immunization to other red cell antigens is always a possibility. **If antibody begins to appear in the circulation before all of the donor red cells have lived out their expected lifespans, the remaining cells will be destroyed by the newly-formed antibody. This clinical situation is called a delayed hemolytic transfusion reaction.** The patient is usually asymptomatic, and the condition is diagnosed when a recently transfused patient exhibits falling hemoglobin in the absence of blood loss. The antibody screen (indirect Coombs test) shows a “new” antibody (i.e., one that was not detectable prior to the transfusion in question), and the direct Coombs test is positive for IgG (plus or minus C3) as long as donor cells remain in the circulation. Delayed hemolytic reactions are rare, occurring only once for every 20,000 to 30,000 units of blood transfused, but occur more commonly in frequently transfused patients.

G. Transfusion-Associated Acute Lung Injury (TRALI)

Transfusion-associated acute lung injury, or TRALI, is currently the most common cause of transfusion-related death. TRALI is a form of severe lung injury that occurs within a few hours of transfusion. Vulnerable transfusion recipients typically have an underlying condition such as sepsis or pneumonia that causes “priming” of their neutrophils. It is thought that these primed neutrophils release their potent microbicidal arsenal upon exposure to activating substances in the transfused blood product. Examples of activating substances may include anti-HLA antibodies (present in about 80% of cases) that developed in the donor as a result of prior pregnancy, or lipids that have accumulated during storage. The resulting endothelial damage causes leakage of fluid into the pulmonary airspace and a form of non-cardiogenic pulmonary edema. TRALI is treated supportively, with oxygen and (if needed) assisted ventilation. It must be distinguished from transfusion-associated volume overload, which can also cause pulmonary edema but which is treated with diuretics. The mortality rate of TRALI is about 5%. It is most likely to occur after transfusion of fresh frozen plasma and platelet concentrates.

H. Other Transfusion Reactions Not Involving Red Cells

Fever not due to red cell antigen-antibody reactions is the most common transfusion reaction (up to 5% of transfused units) and may be stimulated by foreign leukocytes, platelets, or plasma components. **Urticaria** may be caused by reactions to allergens ingested by the donor, hence donors are asked to come to the blood center fasting. Rarely, a white cell agglutination reaction will be accompanied by dyspnea and pulmonary infiltrates. **Anaphylaxis** may be caused

by anti-IgA antibodies in a patient deficient in IgA. The anaphylactic reaction caused by IgA in transfused blood can be avoided by giving washed red cells.

I. Volume-Related Transfusion Reactions

Finally, patients whose entire blood volume is replaced with donor blood within a period of 12 to 24 hours may develop special problems, generally related to changes that occur in the donor blood during storage. Such complications may include:

- hypocalcemia due to citrate overload (citrate binds free Ca^{++}),
- hyperkalemia (potassium leaked from stored red cells),
- acidosis (hydrogen ions in storage solution plus glycolytic products),
- alterations in oxygen transport (2,3 BPG is depleted in stored blood),
- bleeding from dilution of clotting factors,
- respiratory insufficiency due to volume overload

VI. Summary

Transfusion of the components of blood—red cells, platelets, whole plasma, and purified plasma proteins—is important for treatment of a wide variety of diseases. Risks of transfusions fall into two major categories—immunologic reactions and transmission of infection. A working knowledge of the relative importance of various red cell antigens and of transfusion-transmitted diseases is required for the practice of medicine.

CHAPTER 7

IMMUNE HEMOLYSIS

Key Concepts:

- Pathophysiology, diagnosis, and approach to treatment of autoimmune and drug-induced immune hemolytic anemia.
- Pathophysiology, diagnosis, and principles of treatment and prevention of hemolytic disease of the newborn

Learning Objectives:

1. Describe the pathophysiology and laboratory findings associated with warm and cold antibody-mediated immune hemolytic anemia and the principles of treatment of each condition.
2. Describe the direct antiglobulin test and be able to use the results of this test to help diagnose the various forms of immune hemolytic anemia.
3. Describe three mechanisms of drug-induced hemolysis.
4. Describe the pathophysiology of hemolytic disease of the newborn and explain how this condition is prevented and treated.
5. Be able to predict the risk of hemolytic disease of the newborn using information about antigens present on the red cells of mother and fetus, and the clinical history.

I. Introduction

A. Definition

Immune hemolysis is antibody-mediated destruction of circulating red cells. Immune hemolysis can be classified broadly into isoimmune (immune destruction of foreign red cells), autoimmune, and drug-induced immune reactions. This chapter discusses all three.

B. Interpretation of the Direct Antiglobulin (Coombs) Test

An essential requirement for classifying a hemolytic anemia as immune is the demonstration of an antibody or complement on the erythrocyte membrane. Antibody or complement on the erythrocyte can be detected by the direct antiglobulin (Coombs) test. As described in Chapter 6, the antiglobulin reagent agglutinates red cells by attaching simultaneously to antibody or complement molecules on two or more erythrocytes.

1. False Negatives

Most patients with antibody-mediated hemolysis have a positive direct antiglobulin test. However, false negative tests do occur. The Coombs reagent cannot detect fewer than 100 to 500 molecules of antibody or C3 per erythrocyte. A lower density of antibody or C3 molecules can at times produce hemolysis but a false-negative direct antiglobulin test. (For reference, fewer than 35 molecules of IgG can be found on the normal erythrocyte.). Two percent to five percent of patients with immune hemolytic

anemias have a falsely negative direct antiglobulin reaction, and special methods are required to detect the antibody. Immune hemolytic anemia with a truly negative direct antiglobulin test is rare.

2. False Positives

A positive direct antiglobulin test is not proof of immune hemolysis. A false-positive direct antiglobulin test may occur in the presence of coexisting autoimmune disease or drug therapy.

The IgG subclass is an important determinant in autoimmune hemolytic disease. IgG3 antibodies are associated with marked shortening of the erythrocyte life span. IgG1 antibodies are occasionally associated with premature erythrocyte destruction. IgG2 and IgG4 antibodies are rarely associated with shortening of erythrocyte survival. Thus, the presence of IgG2 or IgG4 on the erythrocyte is one explanation for a positive direct antiglobulin reaction in the absence of overt hemolysis.

II. General Mechanisms

A. Extravascular Hemolysis

Extravascular autoimmune hemolysis is much more common than intravascular autoimmune disease. It is caused primarily by IgG autoantibodies and occasionally by IgM antibodies with incomplete complement activation. The antibody class and the presence or absence of the complement component C3b determine the predominant site of extravascular hemolysis. **The spleen is an efficient filter of IgG-coated erythrocytes.** Splenic macrophages have receptors for the Fc fragment of IgG with specificity for IgG1 and IgG3. Some of these macrophages also have receptors for the activated third component of complement, C3b. These receptors act synergistically in binding IgG and C3b, and erythrocytes coated with both IgG and C3b are cleared more efficiently than those coated with IgG alone.

The liver has a relatively small concentration of macrophages with IgG receptors, compared with the spleen, although the **hepatic macrophages have a larger number of receptors for C3b.** Clearance of IgM-coated erythrocytes occurs through partial activation of the complement sequence, attachment of C3b to the erythrocyte membrane, and detection of erythrocyte C3b by the hepatic macrophage receptor. As a result, **the liver is the predominant site of extravascular hemolysis of IgM-coated erythrocytes.**

Red cell survival is proportional to the number of either IgG or IgM antibody molecules per cell. Since the liver receives 30% of the cardiac output (whereas the spleen receives 5%), the liver becomes the major site of hemolysis when large numbers of IgG molecules are present on the cell.

B. Intravascular Hemolysis

Intravascular hemolysis in immune hemolytic anemia requires fixation and complete activation of complement. Although IgM isoantibodies (ABO incompatibility—see chapter 6) predictably cause intravascular hemolysis, IgM autoantibodies usually do not. They most often bind complement only through C3b and the red cells are removed by liver macrophages.

The nature and subclass of the antibody and the frequency and proximity of the antigenic sites are all important determinants of whether hemolysis will be intravascular or extravascular:

- IgM autoantibodies are more likely than IgG antibodies to activate complement to C9, but occasionally IgG can also do so. One molecule of IgM or two molecules of IgG are necessary to completely activate one molecule of complement. In intravascular hemolysis due to IgG, complement activation occurs when two IgG molecules, called a “doublet,” are within 250 to 400 angstroms on the erythrocyte membrane.
- A second determinant of complement activation by IgG is the antibody subclass. IgG4 does not activate complement. The IgG1 and IgG3 subclasses are strong activators of complement, while IgG2 is a weak activator of complement.
- A third determinant of complement activation by IgG is the proximity and number of antigen binding sites on the erythrocyte membrane. There are 10,000-20,000 Rh (D) antigens per red cell, but about 800,000 A and B antigens per red cell. This is another reason why intravascular hemolysis is typical of ABO mismatched red cell transfusions, whereas Rh mismatches usually result in extravascular hemolysis (see Chapter 6).

III. Hemolytic Disease of the Newborn

A. Introduction

There are at least five causes of hemolytic disease of the newborn (HDN):

- hereditary elliptocytosis
- hereditary spherocytosis
- G6PD deficiency,
- alpha thalassemia
- maternal antibody

In this chapter, discussion of HDN is limited to maternal antibody. Maternal IgG antibody may be naturally occurring or due to sensitization by transplacental hemorrhage or previous transfusion. **98% of cases of hemolytic disease of the newborn are due to ABO or Rh(D) incompatibility between the mother and fetus.**

B. Pathogenesis of Hemolytic Disease of the Newborn

HDN is caused by passage of an IgG antibody from the mother across the placenta and attachment of the IgG to a fetal red cell antigen. Hemolysis occurs extravascularly in the spleen, resulting in anemia and the production of unconjugated bilirubin. Unconjugated bilirubin is not dangerous to the fetus because it is cleared by the placenta and metabolized in maternal liver. When hemolysis is severe, red cell precursors proliferate in the liver and spleen and appear in the blood (erythroblastosis fetalis). The liver becomes obstructed and injured by this normoblastic hyperplasia, leading to massive hepatosplenomegaly, edema, and ascites (hydrops fetalis – see Chapter 5). This can lead to fetal or neonatal death.

Jaundice occurring during the early postpartum period in normal infants is called "physiologic jaundice of the newborn," and is due to the inability of the immature liver to conjugate bilirubin due to low levels of glucuronyl transferase. **In an infant born with significant hemolytic disease, extremely high levels of unconjugated bilirubin may accumulate. Indirect**

bilirubin levels greater than 20 mg/dl are associated with neurological damage due to deposition of unconjugated bilirubin in the basal ganglia (kernicterus).

The clinical expression of hemolytic disease of the newborn is dependent on the following variables:

- the concentration of IgG antibodies that cross the placenta into the fetal circulation;
- the capacity of the fetal monocyte-macrophage system to destroy antibody-coated erythrocytes;
- the ability of the fetal bone marrow to increase red cell production;
- the ability of the neonatal liver to synthesize glucuronyl transferase (i.e., hepatic maturity).

C. Hemolytic Disease of the Newborn Due to ABO Incompatibility

Maternal-fetal ABO group incompatibility is the most common cause of hemolytic disease of the newborn. It is almost always seen in infants born to type O mothers, since naturally occurring *IgG* anti-A and anti-B antibodies occur only in the sera of group O individuals. Type A, B, and AB individuals make only *IgM* anti-A and anti-B, which is too large to cross the placenta. **Most episodes of hemolytic disease due to ABO incompatibility are asymptomatic or mild; only ten percent of severe hemolytic disease is due to ABO incompatibility.** The disease is mild because

- 80% of the population secrete soluble ABH antigen, which binds much of the antibody, thereby keeping it away from the red cell
- the ABO blood group antigens are not well-developed at birth

Since the offending antibodies are usually naturally occurring, no primary immunization is necessary, and hemolytic disease of the newborn may occur with the first pregnancy that is ABO-incompatible. Unlike Rh(D) hemolytic disease, the development of ABO-incompatible hemolytic disease of the newborn with one pregnancy is of no prognostic value in predicting the incidence or severity of ABO-incompatible hemolytic disease with subsequent pregnancies.

D. Hemolytic Disease of the Newborn Due to Rh(D) Incompatibility

In contrast to ABO hemolytic disease, Rh(D) incompatibility requires prior sensitization and is likely to cause severe hemolysis. Although small volumes (0.1 ml) of fetal erythrocytes may cross the placenta during pregnancy, such quantities are usually not enough to immunize the mother. Hemolytic disease, therefore, does not usually occur with the first pregnancy. However, significant transplacental hemorrhage may occur at labor and delivery so that immunization can occur, and subsequent pregnancies with Rh(D) positive fetuses are at risk for hemolytic anemia. Primary immunization can also occur when an Rh(D) negative woman aborts an Rh(D) positive fetus. Since immunization is more likely to occur with large numbers of Rh(D) positive erythrocytes, transfusion of Rh(D) positive erythrocytes is more likely to immunize an Rh(D) recipient than is a pregnancy with an Rh(D) positive fetus. About 15% of Rh negative mothers with Rh positive babies become immunized to the Rh(D) antigen during labor and delivery.

Following primary immunization, the small numbers of erythrocytes that cross the placenta during a subsequent pregnancy are sufficient to induce a secondary immune response. The secondary immune response results in an increase of the maternal Rh(D) antibody titer. The appearance of Rh(D) antibody in the maternal circulation is an important prognostic sign: all

future pregnancies with Rh(D) positive fetuses will be affected, and the severity of the disease tends to be progressive with each Rh(D) pregnancy.

ABO antigens have an important effect on Rh(D) hemolytic disease of the newborn (Table 7.1). When pregnancy occurs with an ABO-compatible, Rh(D) positive fetus, the frequency of immunization is about 15% per pregnancy. However, when pregnancy occurs with an ABO-incompatible, Rh(D) positive fetus, the frequency of immunization is about 3% per pregnancy. This protective effect of ABO incompatibility occurs because ABO-incompatible fetal erythrocytes entering the maternal circulation are rapidly destroyed intravascularly by complement-fixing anti-A or anti-B alloantibodies before they reach the monocyte-macrophage system, where primary immunization against Rh antigens could occur.

Table 7.1 Effect of ABO type on likelihood of Rh sensitization

| Fetus | Mother | Fate of fetal RBC | % Immunization |
|--|---|--|----------------|
| O Rh positive → O or A Rh positive → O or B Rh positive → A, B, O or AB Rh positive → | O Rh negative A Rh negative B Rh negative AB Rh negative | Persist in maternal circulation up to 120 days | 15% |
| A, B, or AB Rh positive → B or AB Rh positive → A or AB Rh positive → | O Rh negative A Rh negative B Rh negative | Destroyed immediately by anti-A or anti-B antibodies | 3% |

E. Treatment

Treatment is intended to prevent complications caused by anemia and hyperbilirubinemia. In mild hemolytic disease of the newborn, as is often seen with ABO incompatibility, delivery effectively interrupts the source of maternal antibody. No specific therapy is necessary, since the residual antibody-sensitized erythrocytes are cleared by macrophages, and the infant's bone marrow and liver are able to compensate for the mild degree of hemolysis.

Simple transfusions may be used in those infants with only mild anemia and mild hyperbilirubinemia. Severe hemolytic disease of the newborn (cord blood Hb <12 g/dl and cord bilirubin > 5 mg/dl) is treated with **exchange transfusions**, which correct the newborn's anemia and help prevent kernicterus by removing unconjugated bilirubin. Additionally, maternal antibody is removed from the newborn's circulation, decreasing hemolysis. The transfused Rh-negative cells are of course not subject to destruction by any residual maternal antibody.

Phototherapy is used as adjunctive therapy for hyperbilirubinemia. Blue-violet and yellow-green light slowly oxidizes bilirubin pigments in the skin to water soluble compounds that are excreted in the urine.

Erythroblastosis fetalis should be suspected in pregnant women when there is a history of neonatal hemolytic disease or hydrops fetalis following a previous pregnancy, or when there is sensitization to the Rh(D) antigen. Amniocentesis should be performed when rising antibody titers are found on serial antibody screens (indirect antiglobulin or indirect Coombs test – see Chapter 6). In a sensitized mother, the level of bilirubin in the amniotic fluid is directly related to the severity of erythroblastosis in the fetus. Intrauterine transfusion may prevent hydrops fetalis in a fetus with erythroblastosis that is younger than 34 weeks' gestation. Beyond 34 weeks' gestation, when pulmonary maturity is suggested by amniocentesis, early delivery may be necessary to interrupt severe erythroblastosis fetalis.

F. Prevention

Rh(D) sensitization is preventable. Rh(D) negative women of child-bearing age should not be transfused with Rh(D) positive erythrocytes except in life-threatening circumstances.

Routine prenatal screening of pregnant women should include ABO and Rh(D) typing. An antibody screen of the mother's serum should be done at both two to three months' and seven to eight months' gestation. The Rh type of the fetus should be determined at birth. **All unsensitized Rh(D) negative women should be treated with Rh immunoglobulin (RhoGam®) at around 28 weeks of gestation. A second dose should be given within 72 hours of delivery if the child is Rh positive.** Rh immunoglobulin should also be given to all Rh(D) negative women after an abortion, miscarriage, amniocentesis or any other event that might introduce fetal blood into the maternal circulation.

The mechanism of action of Rh immune globulin is not known. Simple antigen blockade explains only some of the effect. There is also evidence that a central inhibition of the immune system by IgG immune complexes blocks the primary immune response.

Rh immune globulin prevents immunization in 90% of the women at risk. The remaining 10% of women who are therapeutic failures probably have had significant transplacental hemorrhages in the third trimester. The measures described have reduced fetal death due to antibody-mediated hemolytic disease 90% in recent years.

IV. Autoimmune Hemolytic Anemia

A. Incidence

The annual incidence of autoimmune hemolysis is approximately 1/80,000. While there does not always appear to be a genetic basis to the development of autoimmune hemolysis, there are patients with autoimmune hemolytic anemia who have family histories of other autoimmune diseases.

B. Etiologic Classification of Autoimmune Hemolytic Anemia

The autoimmune hemolytic anemias are classified according to the presence or absence of an underlying disease process (primary vs. secondary), and according to the thermal optimum of the autoantibody (warm vs. cold antibody). In approximately one third of all autoimmune hemolytic anemias, no underlying disease is identified. Secondary autoimmune hemolytic anemias are associated with malignancies (especially lymphomas), certain infections, rheumatologic

disorders, and drugs. **Warm autoantibodies are predominantly IgG, with optimum antibody activity at 37 °C. Cold autoantibodies, usually IgM, are more active at 0 to 4 °C or at room temperature than at 37 °C.**

C. Pathogenesis and Clinical Description of Warm Autoimmune Hemolysis

IgG autoimmune hemolytic anemia is characterized by a positive direct antiglobulin test and extravascular hemolysis. The antibody is active in a thermal range of 25 to 37 °C.

Complement is not usually activated, or only partial complement activation through C3 occurs. The antibody may show general affinity for the Rh group of antigens. IgG antibody has no effect on the red cell *in vitro*. Damage comes from contact with macrophages *in vivo*.

Hemolysis occurs predominantly in the spleen.

The clinical manifestations of warm-type autoimmune hemolytic anemia vary according to the severity of the anemia, and the presence of underlying heart or lung disease. Weakness, malaise, dyspnea on exertion, and lightheadedness are usually attributable to anemia. Congestive heart failure or angina pectoris may be seen in patients with cardiovascular disease. The blood smear shows polychromatophilic macrocytes (shift cells) and, in many cases, spherocytes. Spherocytes are the result of membrane injury from contact with macrophages. Red cell agglutination on the blood smear, a common finding with cold-reacting antibodies, is not seen in warm autoimmune hemolysis.

D. Pathogenesis and Clinical Description of Cold Autoimmune Hemolysis

Most cold autoantibodies are of the IgM class. The clinical manifestations of cold autoimmune hemolytic anemia depend on the amount of antibody coating the erythrocytes, the thermal amplitude of the antibody, and the ability of the antibody to fix complement. A minimum antibody concentration of about 20 IgM molecules per cell is required to cause increased erythrocyte clearance in humans. If the upper thermal limit of antibody activity is less than 28 °C, obvious hemolysis is unlikely. **Clinically significant hemolysis and erythrocyte agglutination occur when the thermal limit is above 28 °C. Hemolysis may be intravascular (if complement activation is complete) but is more commonly extravascular.** When hemolysis is extravascular, it occurs primarily in the liver, since C3b receptors are expressed mainly by hepatic macrophages. Table 7.2 compares warm and cold red cell antibodies.

TABLE 7.2. WARM VS.COLD ANTIBODIES

| Type | Warm | Cold |
|---|--|--|
| Immunoglobulin | IgG | IgM |
| Temperature at which antibody is usually active | 37 °C | <32 °C |
| Complement activation | none or partial | partial, rarely complete |
| Antibody specificity | broad specificity Rh system | I, i |
| Predominant site of erythrocyte destruction | spleen | liver or intravascular |
| Common underlying causes (if any) | drugs, B lymphocyte malignancies collagen vascular disease | infections, B lymphocyte malignancies |

E. The Cold Agglutinin Syndrome

The cold agglutinin syndrome is an autoimmune hemolytic anemia due to an IgM antibody.

Most IgM cold agglutinins adhere to erythrocyte membranes at less than 25 °C, and clinical hemolysis is unusual because temperatures in the extremities do not usually fall below 30 °C. However, some cold agglutinins are active up to body temperature and cause hemolysis even at low antibody density. In such cases, agglutination is often apparent on the blood smear. When complement is completely activated, intravascular hemolysis occurs. In most instances, however, incomplete complement activation through C3b occurs, with extravascular hemolysis occurring predominantly in the liver. **The direct antiglobulin test is typically positive for C3 but negative for IgG.**

Raynaud's phenomenon is occasionally seen in association with the cold agglutinin syndrome. When the thermal range of a cold antibody extends up to 28 to 32 °C., the skin temperature of the extremities may fall to within the active range of the antibody and agglutination of erythrocytes occurs within the capillaries. Vascular sludging results, so that impaired circulation to the affected extremities occurs. Signs and symptoms include pain, paresthesias, and cyanosis of the extremities.

The cold agglutinin syndrome may be idiopathic but is seen following infectious disease (*Mycoplasma pneumoniae* and infectious mononucleosis), and in association with malignant lymphomas. Most idiopathic cold agglutinins, including those associated with mycoplasma infections, are directed against a specific antigen (the I antigen) on the erythrocyte membrane. Cold agglutinins seen with infectious mononucleosis and some malignant lymphomas are directed against the i antigen, a chemical precursor of the I antigen that is found mainly on fetal

and infant blood cells (in older children and adults, i is converted to I by a developmentally-regulated glycosyltransferase). Hemolysis is therefore rarely associated with anti-i, since the i antigen is not well expressed on adult erythrocytes. Cold agglutinins associated with lymphomas are typically monoclonal.

F. Drug-Induced Hemolytic Anemia

Drugs cause 16% to 18% of all cases of acquired immune hemolytic anemia. **The essential features of drug-induced immune hemolysis are a positive direct antiglobulin test with evidence of increased erythrocyte destruction, and a clinical course that can be correlated with current or recent drug therapy.**

Drug-induced positive direct antiglobulin tests are subclassified according to four underlying mechanisms: immune-complex adsorption to erythrocytes ("innocent bystander"), drug adsorption onto erythrocytes (hapten), membrane modification, and true autoimmunity (suppressor cell). Membrane modification is not associated with hemolysis. These four mechanisms are outlined in Table 7.3.

Table 7.3

MECHANISMS OF DRUG-INDUCED POSITIVE DIRECT ANTIGLOBULIN TEST.

| Mechanism | Prototype Drugs | Clinical Findings | Antibody Class | Antiglobulin Test |
|-----------------------------------|-----------------------------|---|--|--|
| immune adsorption | quinidine | Acute I.V. hemolysis. Hemoglobinemia; Hemoglobinuria; Thrombocytopenia may occur; recovery with withdrawal of drug. | IgM Fixes complement completely. | Drug and antibody in patient's serum form a complex that then attaches to the erythrocyte. |
| Drug adsorption onto erythrocytes | penicillins, cephalosporins | Extravascular hemolysis occurs in 1% of patients receiving >10 million units I.V. penicillin. | IgG. May rarely incompletely fix complement. | Requires drug-coated RBC and patient's serum as a source of antibody |
| membrane modification | cephalosporins | No Hemolysis | IgG and other non-specific proteins | Requires drug-coated RBC and patient's serum as source of antibody. |
| true autoimmunity | alpha-methyl dopa | Extravascular hemolysis in 0.8% | IgG. May rarely incompletely fix complement; antibody commonly has Rh specificity. | Presence of drug not necessary; requires normal erythrocytes and patient's serum as source of antibody |

Adapted from Garratty G, Petz L: Drug-induced hemolytic anemia. *Am J Med* 58:398-407, 1975.

1. Immune-Complex Adsorption to Erythrocytes

In the immune-complex type (Fig. 7.1), drug and anti-drug antibody combine and are adsorbed onto the erythrocyte membrane, which is an "innocent bystander."

The prototypic drug is quinine. The anti-drug antibody in the serum usually is of the IgM class and fixes complement through C9, causing **intravascular hemolysis**. The direct antiglobulin test is positive for C3. Hemolysis can occur after ingestion of a small quantity of drug. Drugs can cause thrombocytopenia by a similar mechanism (see chapter 12).

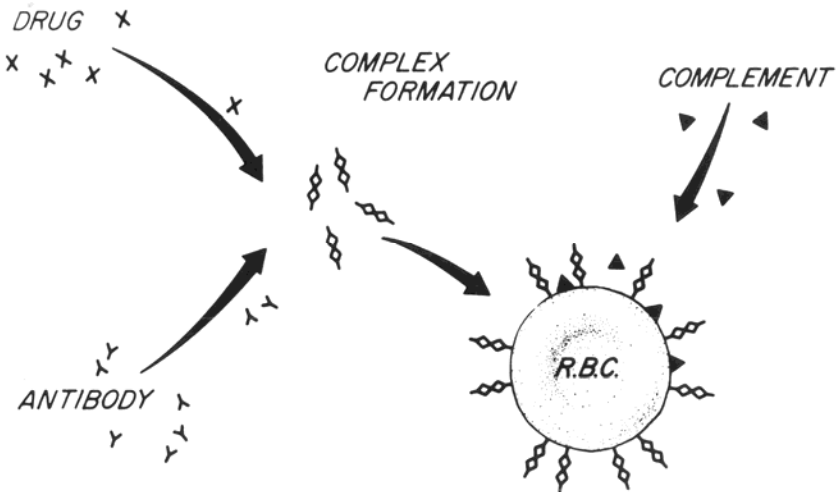


Figure 7.1. Immune-complex type of drug-induced hemolysis. The antibody is usually IgM and fixes complement, leading to intravascular hemolysis. (From Garratty G, Petz L: Drug-induced immune hemolytic anemia, *Am J Med* 58:398).

2. Drug Adsorption onto Erythrocytes

Immune hemolysis may occur due to drug adsorption onto erythrocytes (Fig. 7.2). The prototypical drug is penicillin, which readily reacts with tissue proteins to form haptenic groups, and can be detected on the erythrocyte membranes of all patients receiving large intravenous doses. **This type of drug-induced hemolytic anemia is characterized by an IgG antibody, extravascular hemolysis, and complete recovery following cessation of drug therapy.**

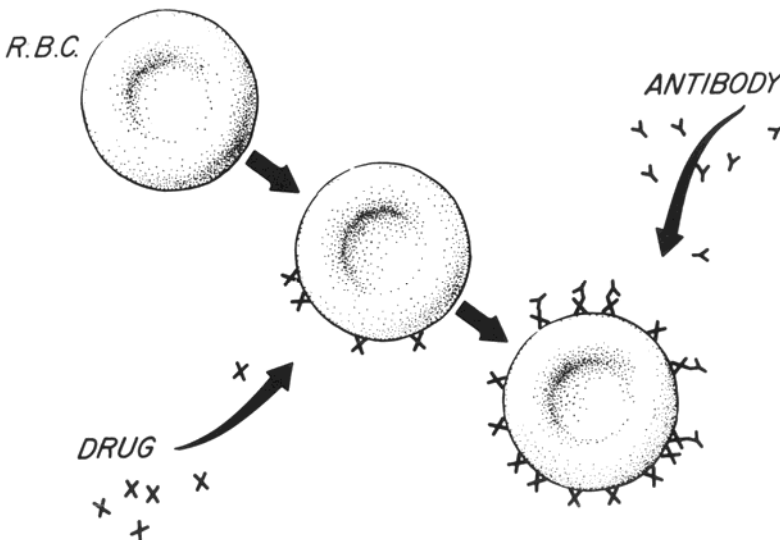


Figure 7.2. Haptenic type of drug-induced hemolysis. The antibody attaches to the drug bound to the red cell membrane. (From Garratty G, Petz L: Drug-induced hemolytic anemia, *Am J Med* 58:398).

3. Membrane Modification

A positive direct antiglobulin test may develop, but hemolysis does not occur when erythrocyte membranes are modified by cephalosporins (Fig. 7.3). This leads to non-immunologic (non-specific) adsorption of plasma proteins by the erythrocyte. The direct antiglobulin test is positive when antisera to various serum proteins are used.

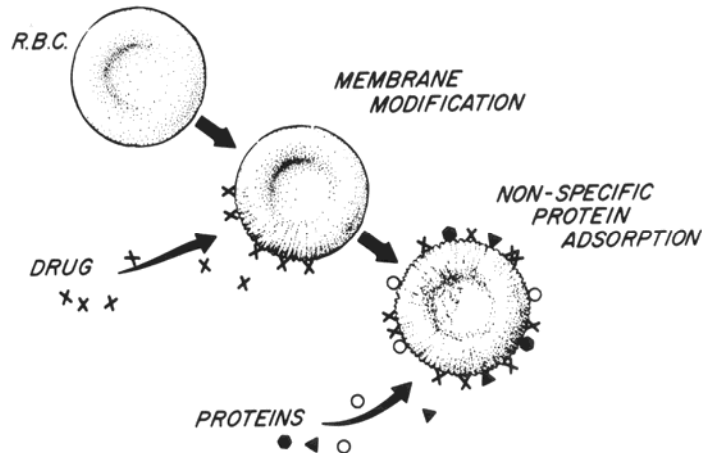


Figure 7.3. Membrane modification type of drug-induced immunoglobulin adherence reaction. Hemolysis does not occur in this nonspecific reaction. (From Garratty G, Petz L: Drug-induced hemolytic anemia, *Am J Med* 58:398).

4. True Autoimmunity

Drugs can also cause true autoimmune hemolytic anemia. The prototype drug is the antihypertensive drug alpha-methyldopa (Aldomet). Alpha-methyldopa induces the production of an IgG antibody that commonly has Rh antigen specificity. After discontinuation of alpha-methyldopa, the direct antiglobulin test becomes negative in one month to two years. Recent investigations suggest that alpha-methyldopa alters the immune system by inhibiting suppressor T cell function, which results in unregulated autoantibody production by B cells in affected patients. A number of other drugs have also been reported to cause true autoimmune hemolysis.

G. Treatment of Autoimmune Hemolytic Anemia

In most secondary autoimmune hemolytic anemias, successful treatment of the underlying disease will control the hemolytic anemia. Drug-induced immune hemolysis usually responds to withdrawal of the offending medication.

Warm-type IgG immune hemolysis often responds to corticosteroid therapy (prednisone).

Corticosteroids act by two mechanisms: an immediate decrease in macrophage Fc-gamma receptors and a slower decrease in the production of the abnormal IgG antibody.

Splenectomy is also effective in IgG immune hemolysis, since it removes both a site of red cell destruction and a major site of antibody production. Splenectomy is reserved for patients who fail to respond to corticosteroids, and for those patients who require prolonged therapy with corticosteroids to maintain an adequate hematocrit. Various forms of immunosuppressive therapy, including the anti-B-cell monoclonal antibody rituximab, are used in patients resistant to corticosteroids and splenectomy.

Corticosteroids are less effective in cold-type IgM immune hemolysis, especially when the hemolysis is intravascular. **Splenectomy is of little benefit because clearance of IgM-coated erythrocytes occurs preferentially in the liver.** Plasmapheresis may provide temporary improvement in IgM immune hemolysis until more effective medical therapy can be initiated. Recently it has been shown that the monoclonal anti-B-cell antibody rituximab is often effective in treating cold agglutinin disease.

Transfusions may become necessary when serious anemia is present. This often requires administering blood even though the cross match indicates incompatibility. The major risk of transfusion in this circumstance is that clinically significant alloantibodies may not be detected because they are masked by the presence of autoantibodies. When compatible donor erythrocytes are transfused, however, the transfused cells will usually have a survival rate comparable to that of the patient's own cells. Patients with cold autoantibodies should be transfused with blood that has been run through a warming coil to raise the temperature above 30 °C.

V. Summary

Immune hemolysis is mediated by the presence of antibody or complement, or both, on the erythrocyte membrane. Antibody production may occur in the absence of identifiable causes (idiopathic), or may be secondary to an underlying disease or the use of specific medications. Factors that determine whether an antibody causes hemolysis and the severity of any hemolysis include

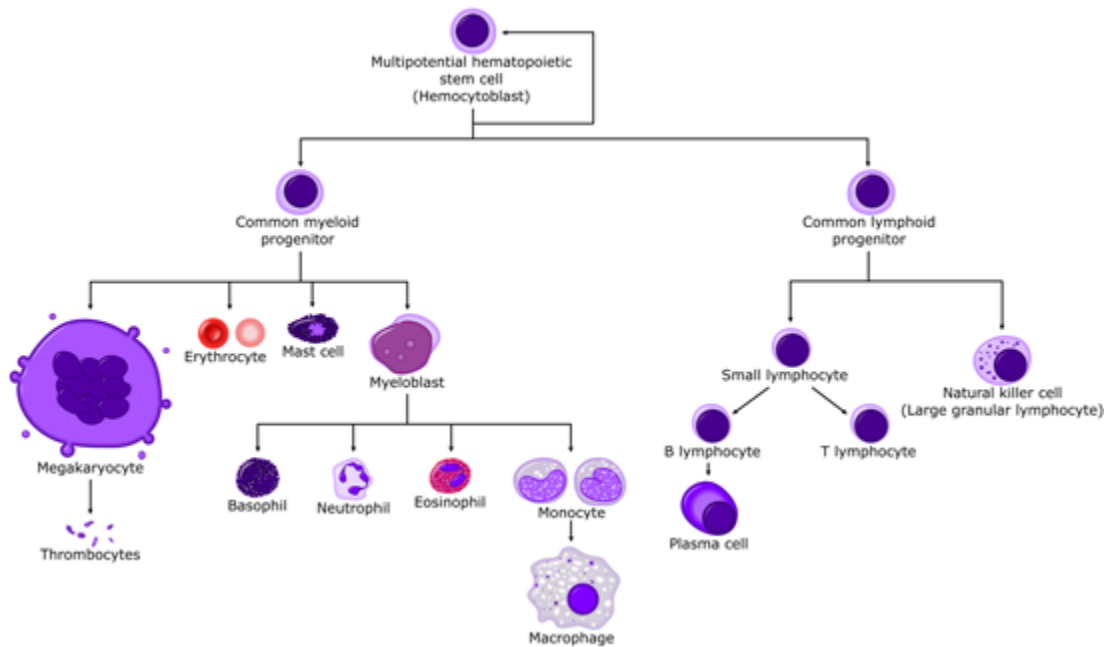
- the immunoglobulin class and subclass of the antibody
- the ability of the antibody to attach complement to erythrocytes and activate the complement cascade
- the antigenic specificity of the antibody
- the concentration of the antibody
- the number of macrophages that recognize specific antigen-antibody complexes of components of complement on the erythrocyte membrane.

INTRODUCTION TO THE HEMATOLOGIC MALIGNANCY (WHITE CELL) SECTION

The “white cell” section of this course (Chapters 8-10) is mainly about the malignancies that arise from hematopoietic progenitor cells. The subject can be very intimidating because there are so many individual malignancies to try to keep track of. This is an attempt to provide you with a framework.

Step 1.

Review the marrow stem cell system carefully because every hematologic malignancy arises from one or another of these normal counterparts.



Step 2.

Divide the malignancies into:

- A. Those of myeloid origin (Chapter 8)
- B. Those of lymphoid origin (Chapter 9)
- C. Those of plasma cell origin (Chapter 10)
- D. The acute leukemias (Chapter 8)

(Although acute leukemias are either of lymphoid or myeloid origin, they are all characterized by a paucity of mature cells and have very similar pathophysiology, i.e., anemia, thrombopenia, and neutropenia, with rapid progression and death from bleeding or infection if untreated.)

Then try to outline the basic characteristics of each group and contrast it with the characteristics of each of the other groups. For example, disorders of B lymphocytes (CLL or Non-Hodgkin's lymphomas) have big lymph nodes and often have poor antibody production. Thus, bacterial infection is a significant clinical problem. In contrast, chronic disorders of myeloid origin such as polycythemia vera or essential thrombocytosis primarily involve the marrow. They have normal antibody production and adequate numbers of granulocytes so that infection is rarely a problem. Their symptoms are related to high blood levels of red cells or platelets causing hyperviscosity and clotting problems.

Step 3.

Remember that normal marrow stem cells both proliferate and mature. Ask yourself whether the cells of the malignancy you are considering are proliferating without maturing or proliferating and maturing:

- If proliferating but making no mature cells, the process is “acute,” e.g., acute leukemia in which “blasts” predominate. Disorders in which there are few or no mature cells tend to progress quickly (because immature cells are prone to rapid proliferation) and are rapidly fatal if untreated.
- If proliferating, but the proliferating cells become relatively normal mature cells, the process is “chronic,” e.g., polycythemia vera or chronic lymphocytic leukemia. Patients with these disorders may live a long time, sometimes with little or no treatment.
- Some disorders cannot be put into either of the above categories. For example, in the myelodysplastic syndromes, there is proliferation with abnormal differentiation. In such cases the clinical picture will depend on the number and function (or lack thereof) of the cells that are produced.

Step 4.

Remind yourself what the normal counterpart of the malignant cell does for a living. Then ask yourself if the tumor cell does it in excess or whether it loses the capacity to perform that function. Ask yourself how that is reflected in the pathophysiology of the disease. For example, in multiple myeloma, the normal counterpart is the mature plasma cell. Mature plasma cells secrete immunoglobulins. The clone of malignant plasma cells secretes excessive amounts of a single non-functional immunoglobulin, which may cause a legion of problems. At the same time, it suppresses normal immunoglobulin production, which makes the patient susceptible to bacterial infection.

We hope this helps!

CHAPTER 8

ACUTE LEUKEMIA, MYELOPROLIFERATIVE AND MYELOYDYSPLASTIC SYNDROMES

Key Concepts:

Pathophysiology, pathology, clinical and diagnostic features of:

- Acute myelogenous leukemia (AML) and acute lymphoblastic leukemia (ALL)
- Myeloproliferative disorders (chronic myelogenous leukemia, polycythemia vera, essential thrombocytosis, and myelofibrosis)
- Myelodysplastic syndromes

Learning Objectives:

Acute Leukemia

1. Define leukemia and describe the epidemiology, pathophysiology, morphologic findings, and laboratory and clinical manifestations of AML and ALL.
2. Compare and contrast childhood and adult acute leukemias in terms of biology and prognosis.
3. Describe the role of cytogenetic analysis in the evaluation of leukemias.
4. Describe the principles of treatment of acute leukemia.

Chronic Myelogenous Leukemia

1. Describe the molecular basis and pathophysiology of chronic myelogenous leukemia (CML).
2. Describe the morphologic findings, natural history, cytogenetics, laboratory and clinical manifestations, and treatment of CML.
3. Be able to distinguish CML from benign causes of leukocytosis.

Myeloproliferative Disorders

1. Define “myeloproliferative disorder” and describe the morphologic findings, natural history, laboratory and clinical manifestation, and treatment of the chronic myeloproliferative disorders. Distinguish myeloproliferative disorders from benign causes of erythrocytosis and thrombocytosis.
2. Describe the role of the JAK2 mutation in the pathophysiology of the chronic myeloproliferative disorders.

Myelodysplasia

1. Define “myelodysplasia.”
2. Describe the epidemiology, morphologic findings, and laboratory manifestations of myelodysplastic syndromes.

I. Introduction

This chapter covers the following topics:

A. Distinguishing between acute and chronic hematologic malignancies

- Acute leukemias progress rapidly over a period of days to weeks and therefore cause symptoms within a short time.
- Chronic conditions, such as myeloproliferative diseases, progress slowly over months to years and are often asymptomatic or minimally symptomatic at the time of presentation.

B. Distinguishing between myeloproliferative disease and myelodysplastic syndrome

- In **myeloproliferative diseases**, there is **over-production** of one or more cell lines, and differentiation is normal or nearly normal.
- **Myelodysplastic syndromes** are characterized by the **under-production** of one or more cell lines in conjunction with ineffective erythropoiesis and abnormal cell differentiation.

C. Basic principles of treatment and supportive care for patients with acute leukemia

D. Distinctive molecular, pathologic, and clinical features of individual diseases including:

1. acute myelogenous leukemia
2. acute lymphoblastic leukemia
3. chronic myelogenous leukemia
4. polycythemia vera
5. primary myelofibrosis
6. essential thrombocytosis
7. myelodysplastic syndromes.

II. Pathophysiology

All of the disorders discussed in this section are clonal neoplastic diseases; that is, they are cancers that arise from a single genetically aberrant cell. In this respect, they are no different from any other cancer. Although most cases arise without an obvious cause, a number of environmental agents increase the risk of myeloid malignancies. Two well-known examples are ionizing radiation and benzene. The bone marrow-damaging potential of ionizing radiation is apparent in the annual incidence rate of leukemia in Japanese survivors of the atomic bomb explosions. As shown in Figure 8.1, incidence rates in individuals who were within 1,000 meters and 2,000 meters of the blast rose rapidly over the following years. Those closest to the radiation source were at highest risk to develop leukemia. The incidence of leukemia in these individuals began to rise within a year of the explosion and continued to increase for the next seven years.

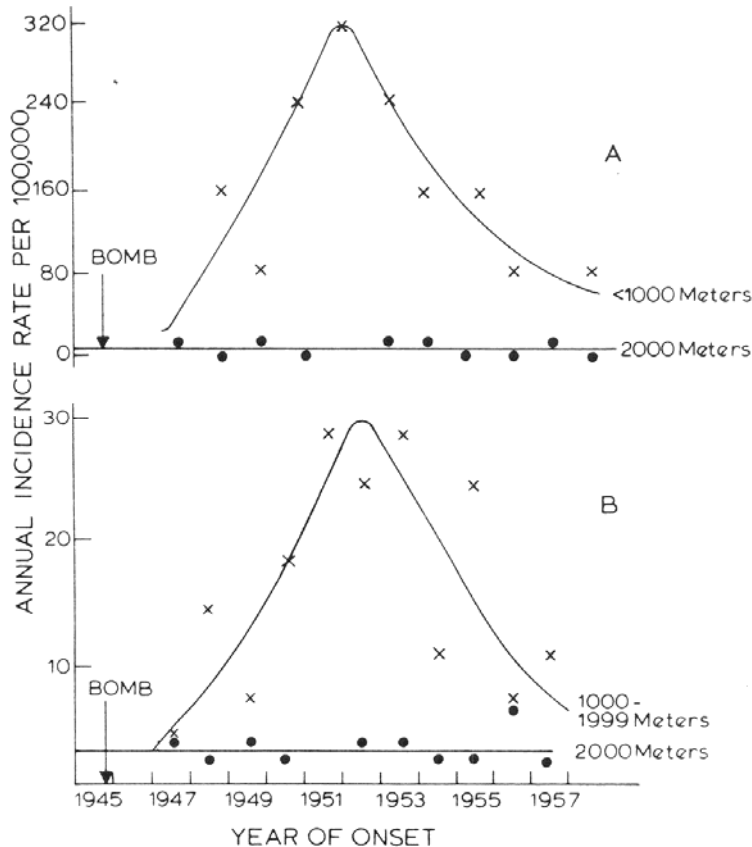


Figure 8.1. Annual incidence rate of leukemia following the atomic bomb explosion among survivors who were residents of Hiroshima at the time of diagnosis. (A) Persons less than 1,000 meters from the hypocenter compared with persons 2,000 meters or more from the hypocenter at the time of explosion. (B) Persons 1,000 to 1,999 meters from the hypocenter compared with persons 2,000 meters from the hypocenter at the time of explosion. AM Lilienfeld. *Foundations of Epidemiology*, Oxford Univ. Press.

Benzene is a ubiquitous environmental carcinogen present in petroleum products and used as an industrial solvent; it is found at many industrial sites. The role of benzene as an important bone marrow toxin was recognized early in the twentieth century with the appearance of blood diseases in individuals working in the rubber, printing, and shoe repair industries where the solvent was commonly employed. Workers who are chronically exposed to greater than 100 parts per million of benzene in inspired air have at least a 50% chance of showing a fall in peripheral blood counts indicative of marrow damage. Benzene-induced bone marrow damage causes genetic damage to hematopoietic cells that can later lead to the development of myeloid malignancies.

Disruption in the genetic control of proliferation and/or differentiation causes myeloid malignancies. In healthy bone marrow, stem cells divide to self-renew and to produce progeny that differentiate into mature blood cells. Their progeny, through a complex genetic program, express various lineage-specific genes that change the characteristics of the cells to those of mature granulocytes, red blood cells, and platelets. The differentiation program that controls the maturation of a cell includes the activation of genes that control apoptosis, which enable the cell to die when it reaches the end of its useful lifespan.

If there is disruption of the genes that control proliferation, but the control of differentiation remains undisturbed, there will be overproduction of normal blood cells (Figure 8.2). Examples of diseases in which this happens are:

- **Chronic Myelogenous Leukemia** – too many white cells
- **Polycythemia Vera** – too many RBCs
- **Essential thrombocytosis** – too many platelets
- **Myelofibrosis** – replacement of marrow by fibrous tissue in response to aberrant clonal proliferation of hematopoietic cells.

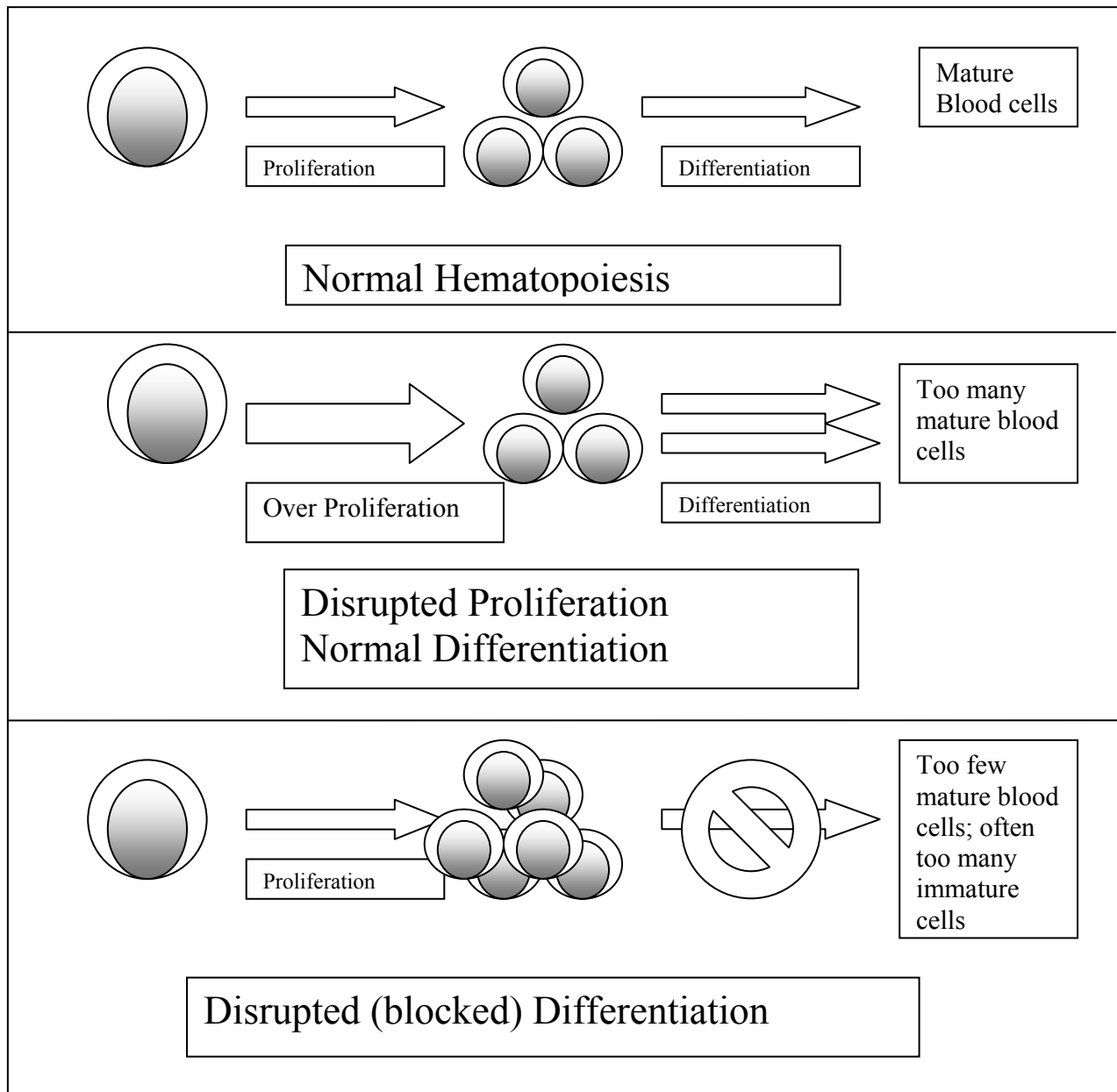


Figure 8.2

If, on the other hand, the differentiation program is blocked, the progenitor cells may proliferate, but if they are unable to mature, only undifferentiated cells are produced. This is what happens in acute leukemia. In acute leukemia, the proliferating but undifferentiated cells usually retain some features of immature **myeloid** or **lymphoid** cells. They are called “blasts” - specifically, myeloblasts (see Chapter 1, Fig. 1.17) or lymphoblasts. In many cases of acute leukemia, there are also genetic changes that encourage proliferation so that the disease progresses quickly.

- **Acute myelogenous leukemia – Accumulation of leukemic blasts with features of myeloid cells.**
- **Acute lymphoblastic leukemia – Accumulation of blasts with lymphoid features.**

Finally, if the bone marrow stem cell is damaged in a way that disrupts but does not block differentiation, blood cell production may be impaired, but not completely inhibited. Myelodysplastic syndromes are diseases characterized by “**ineffective hematopoiesis,**” in which the bone marrow is cellular (often hypercellular) but the peripheral counts are low. The marrow cells exhibit accelerated apoptosis, dying during the process of differentiation. **Differentiation is abnormal,** so that neutrophils, red cells, platelets, and their respective precursors are morphologically and functionally aberrant. Myelodysplasia can present many different ways, and many cases progress over time to acute leukemia.

- **Myelodysplastic syndromes – hematopoietic cells have abnormal differentiation and hematopoiesis is ineffective.**

III. Acute Versus Chronic Blood Myeloid Malignancy

Myeloid malignancies can be divided into two groups:

- Those in which marrow cells are able to grow to maturity (chronic myeloproliferative and myelodysplastic syndromes)
- Those in which precursor cells are unable to differentiate, and accumulate in the bone marrow causing bone marrow failure (acute leukemias).

When there is uncontrolled proliferation of functional mature cells, patients present with symptoms related to the high numbers of normal cells (RBC, WBC, or platelets) in the blood. The disease may be present for months or even years (i.e., **chronic**) before symptoms are recognized. Myeloproliferative disorders are often discovered in the course of routine blood testing in a patient who has no symptoms. When symptoms do occur, they are often related to events caused by the presence of excess numbers of cells in the blood. A classic example is the development of vascular thrombosis associated with an elevated hematocrit in a patient with polycythemia vera. Another example is the development of massive splenomegaly, fevers, and bone pain due to the overproduction of white blood cells in chronic myelogenous leukemia.

In contrast, the presenting features of **acute** leukemias are typically manifestations of the rapid accumulation of undifferentiated (i.e., useless – and in most cases actively harmful) cells in the bone marrow, blood, and other organs. The accumulated undifferentiated cells displace and inhibit residual normal hematopoietic precursors. These patients often present in crisis due to severe pancytopenia caused by bone marrow failure, or to failure of vital organs infiltrated by leukemic cells. The natural history (i.e., untreated course) of these diseases is usually measured in weeks. Common presentations of acute leukemia include sudden fever and collapse due to

sepsis in the setting of neutropenia, or life threatening hemorrhage due to severe thrombocytopenia.

IV. Acute Leukemia

In acute leukemia, progenitor cells are unable to differentiate into healthy blood cells. The differentiation block may occur at different stages of differentiation, so that often the cells bear some resemblance to normal cells of a particular lineage. There are two basic types of acute leukemia:

- acute myelogenous leukemia (AML, also called acute myelocytic or acute myeloblastic leukemia), derived from precursor cells of myeloid lineage
- acute lymphoblastic leukemia (ALL, also called acute lymphocytic leukemia), derived from lymphoid precursors.

Acute myelogenous leukemia is more common as people age. The incidence rises slowly from birth, and more rapidly beyond the age of fifty. The median age of patients with AML is 67; however, the age-specific risk (the risk of AML for a patient at a particular age) continues to rise beyond that age. AML is 50% more common in men. The overall risk of developing acute myelogenous leukemia is approximately equal to the risk of developing cancer of the esophagus, oral cavity, or kidney.

Acute lymphoblastic leukemia is often a disease of childhood, and is the most common form of childhood cancer. The median age at diagnosis for ALL is 13 years and only 40% of cases are diagnosed in adults (20 years or older). There is a second, smaller, rise in incidence after the fourth or fifth decade, but adult ALL is significantly less common than AML. For example, in the sixty-five to sixty-nine year age group there is about one case of ALL per 100,000 patients, versus approximately ten cases of AML.

A. Pathogenesis

Acute leukemia is caused by acquired chromosomal abnormalities that lead to arrested differentiation. Many of these abnormalities have been characterized, and the presence of a specific chromosomal or molecular abnormality can have great prognostic significance (i.e., it can often help predict the likelihood of cure with various therapies). Figure 8.3 shows the relationship between various cytogenetic abnormalities and the prognosis of patients with AML or ALL.

| Pathogenesis | | |
|--|--|-----------------------------------|
| | AML | ALL |
| • Acquired chromosomal abnormalities lead to a disrupted differentiation process | | |
| • Best Prognosis | t(8;21) inv(16;16) t(15;17) | t(12;21) Hyperdiploid |
| • Intermediate Prognosis | Normal cytogenetics Trisomy 8 | |
| • Worst Prognosis | Chromosome 5, 7 11q23 abnl Complex | t(9;22) Hypodiploid t(4;11) |

Figure 8.3

As an example of how a chromosomal abnormality may contribute to the neoplastic phenotype in acute leukemia, consider the case of AML with a translocation between chromosome 8 and 21 (denoted t(8;21)). This form of AML is sometimes called a “core-binding factor (CBF)” leukemia because the translocation affects the CBF complex that controls myeloid differentiation.

Normally, a differentiating myeloid cell reaches a stage at which the transcription factors CBF α and CBF β bind and attract a collection of transcriptional activators that are required for transcription of several genes (including those for IL-3, GM-CSF, and M-CSF) necessary for neutrophil maturation (Figure 8.4). If a maturing myeloid cell has acquired the (8;21) translocation, the normal CBF α gene from chromosome 21 is translocated onto a section of chromosome 8 containing a gene named ETO.

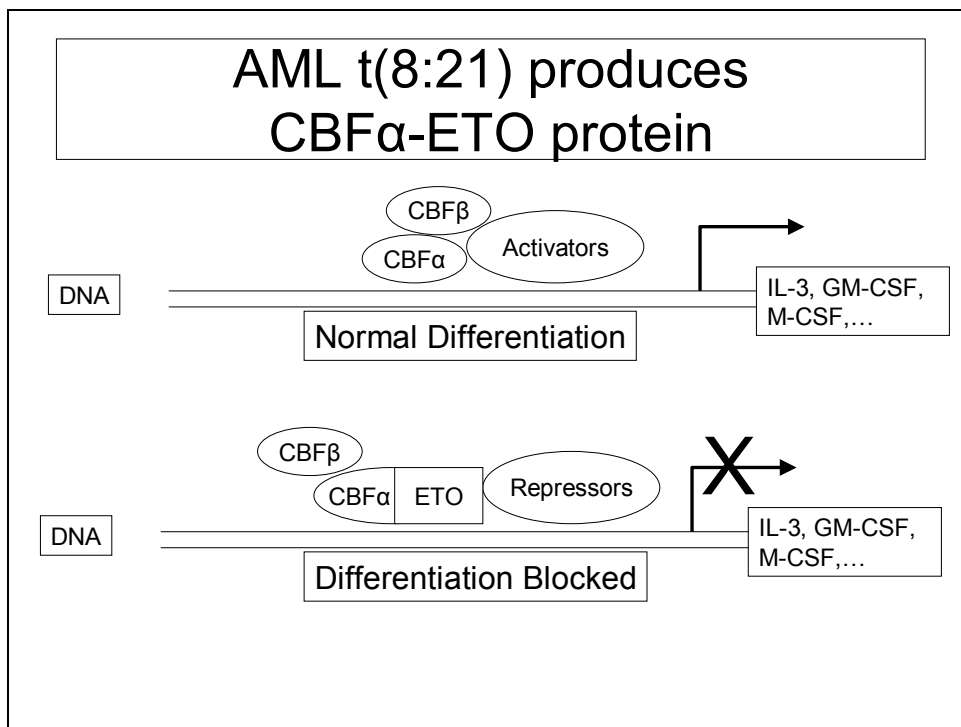


Figure 8.4

This translocation then leads to the transcription of a hybrid CBF α -ETO mRNA that is translated into a chimeric CBF α -ETO transcription factor. This transcription factor has the unique property of being an attractor of transcriptional **repressor** proteins, and effectively blocks the next step in myeloid differentiation. These cells are unable to further differentiate along the neutrophil pathway, but remain capable of cell division and proliferation. A similar pathogenesis has been described for AML with a specific inversion on the 16th chromosome. In this case, the gene for CBF β transcription factor is affected.

The genotype of AML often predicts the phenotype (i.e., the clinical behavior, and in many cases the response to treatment) of the disease.

- In the case of “CBF” leukemias, the response to treatment is generally good and many patients can be cured with high doses of a chemotherapy drug called cytosine arabinoside (cytarabine).
- In contrast to the favorable prognosis of a “CBF” leukemia, forms of AML or ALL with a (9;22) translocation (the “Philadelphia chromosome,” discussed in more detail in the section on chronic myelogenous leukemia) carry a poor prognosis. They are usually incurable with chemotherapy alone, but can sometimes be cured by allogeneic stem cell transplantation.
- Another form of AML, called acute promyelocytic leukemia or APL because the leukemic cells resemble promyelocytes, is associated with a translocation that places the retinoic acid receptor alpha gene on chromosome 17 next to a gene called PML on chromosome 15 (t(15;17)). This form of AML is often associated with a severe form of DIC, but has a relatively favorable prognosis because it responds to treatment with all-trans retinoic acid, which can induce differentiation of the leukemic cells, resulting in remission.

B. Clinical Features of Acute Leukemia

Patients with acute leukemia generally suffer from complications of two related problems – bone marrow failure, and organ dysfunction due to infiltration by proliferating leukemia cells. As the disease progresses in the bone marrow, large numbers of immature cells that are unable to differentiate replace normal bone marrow and suppress normal haematopoiesis. This leads to anemia, neutropenia, and thrombocytopenia (i.e., pancytopenia).

- Anemia causes pallor, fatigue, and exertional dyspnea.
- Neutropenia predisposes to acute and sometimes overwhelming bacterial infections caused by Gram-negative organisms from the gut or Gram-positive organisms from the skin.
- Thrombocytopenia causes bruising and spontaneous bleeding.
- Patients frequently present with bone pain due to intense cell proliferation inside the bone marrow.
- Infiltration by leukemic cells may cause enlargement of lymph nodes or the spleen.
- Leukemic involvement of the meninges and brain may cause neck pain, headache, photophobia, and confusion. CNS involvement is particularly common in ALL.
- A particularly fearsome complication is intracerebral bleeding. Contributing factors to this life-threatening problem include thrombocytopenia, infiltration of cerebral blood vessels by leukemic cells, and in some cases DIC caused by release of procoagulant (clot-making) and profibrinolytic (clot-dissolving) substances from leukemic cells.
- Infiltration in the lungs, liver, and kidneys can cause hypoxia, liver dysfunction, and kidney failure, respectively.
- Leukemic cells may infiltrate the skin and cause skin rash, nodules, and pustules (leukemia cutis).

- Patients with very high numbers (100,000 or more) of circulating blasts may develop a condition called **leukostasis**. In this condition, aggregates of leukemic cells cause vascular congestion, occlusion of small vessels, and ischemia. Patient with leukostasis often present with manifestations of multi-organ dysfunction that include mental status changes, visual changes due to retinopathy, and hypoxia due to pulmonary vascular congestion.

C. Diagnosis and Classification of Acute Leukemia

Because of the dual threats posed by bone marrow failure and organ infiltration, patients with acute leukemia often present in crisis and the diagnosis is often made in the emergency room or intensive care unit. **Laboratory findings typically include pancytopenia, with or without circulating blasts. The diagnosis is confirmed by bone marrow biopsy, or in some cases by analysis of circulating blasts if they are present in large numbers.**

At the time of presentation, it is crucial to distinguish the lineage of the leukemia, either myeloid or lymphoid, since the treatments for these diseases are different. Information that is useful for classifying leukemias includes:

- **Morphology.** A particularly useful finding is the presence of cytoplasmic inclusions called **Auer rods**, which are aggregates of myeloperoxidase-containing granules. If present, Auer rods indicate that the leukemia is of myeloid lineage.

Auer rod



- **Cell surface markers** (determined by flow cytometry) (Fig 8.5). For example, AML cells express surface proteins consistent with their myeloid lineage, such as CD13 and CD33. ALL cells typically express antigens consistent with either B cell or T cell lineage. B cell ALL cells express CD19, CD22, and CD10 while T cell ALL cells express CD7, cytoplasmic CD3, and CD2. Expression of terminal deoxynucleotidyl transferase (TdT, an enzyme necessary for immunoglobulin and T cell receptor gene rearrangement) favors a lymphoid lineage.
- **Immunohistochemical stains** for lineage-specific cytoplasmic enzymes and other molecules. For example, myeloid blasts usually express the enzyme myeloperoxidase.
- **Cytogenetic abnormalities** (see above).
- **Molecular analysis.** Immunoglobulin or T cell receptor gene rearrangements indicate B or T cell lineage, for example (Figure 8.5).

Distinguishing ALL from AML

| Markers | AML | ALL |
|--------------------------|----------|--------|
| CD 13/CD33 | + | - |
| CD19,CD22,CD10,TdT | - | B-ALL |
| CD7,CD3,CD2,TdT | - | T-ALL |
| Auer Rods | +/- | - |
| Immunoglobulin/TCR genes | Germline | Clonal |

Figure 8.5 (TCR = T-cell receptor)

D. Supportive Care

Patients with acute leukemia usually receive aggressive chemotherapy regimens. These drug combinations kill not only the rapidly dividing leukemic cells but normal hematopoietic progenitor cells as well, and so cause temporary bone marrow aplasia. The slowly-dividing hematopoietic stem cells are resistant to the effects of most cytotoxic chemotherapy drugs, and will eventually repopulate the marrow with healthy cells if a sufficient number of leukemic cells have been removed. However, during the period of aplasia, patients are at high risk for life-threatening complications. They are dependent on red cell and platelet transfusions that maintain a minimum hemoglobin (usually 8 grams/dl) and platelet count (usually 10,000/ μ l). It is not unusual for the absolute neutrophil count to fall below 100 cells/ μ l. Severe neutropenia of this magnitude greatly increases the risk for infection. Infections in the setting of chemotherapy-induced neutropenia are typically caused by entry of bacteria from the gut (which is also injured by chemotherapy) or skin into the blood stream. Before the widespread availability of broad-spectrum antibiotics capable of effectively treating Gram-negative infections, sepsis was the most common cause of death in patients treated for leukemia. It is now standard practice to immediately treat febrile (temperature $> 38.2^{\circ}\text{C}$) neutropenic (absolute neutrophil count $< 500/\mu\text{l}$) patients with broad-spectrum antibiotics while waiting for the results of cultures. Patients with neutropenia that persists beyond seven to ten days are also at risk for fungal infections, typically caused by organisms such as aspergillus and candida.

E. Treatment

We treat acute leukemia with repeated rounds of high-dose chemotherapy intended to reduce the leukemia cell burden below the level required for re-growth. This treatment strategy is based on studies performed forty years ago demonstrating that repeated rounds of cytotoxic chemotherapy could reduce the number of highly proliferative cells to a level at which re-growth did not occur (Fig. 8.6). From these studies, we know that:

- Chemotherapy-associated prolongation of life in a leukemic mouse is the result of killing leukemic cells, as opposed to slowing the proliferation of these cells or the selection of a population of cells that grow more slowly.
- In order to cure leukemia, it is necessary to bring the number of leukemia cells below a certain threshold. This threshold number is not known, although it has been shown that a single viable leukemia cell inoculated into a mouse can proliferate and cause death from leukemia.
- The most effective anti-leukemia drugs are cytotoxic and kill a larger fraction of malignant cells than normal cells.
- The most effective drugs are cycle-specific agents that attack cells during DNA synthesis (S phase). These drugs are also toxic to other rapidly dividing cells such as those in the gut and skin.

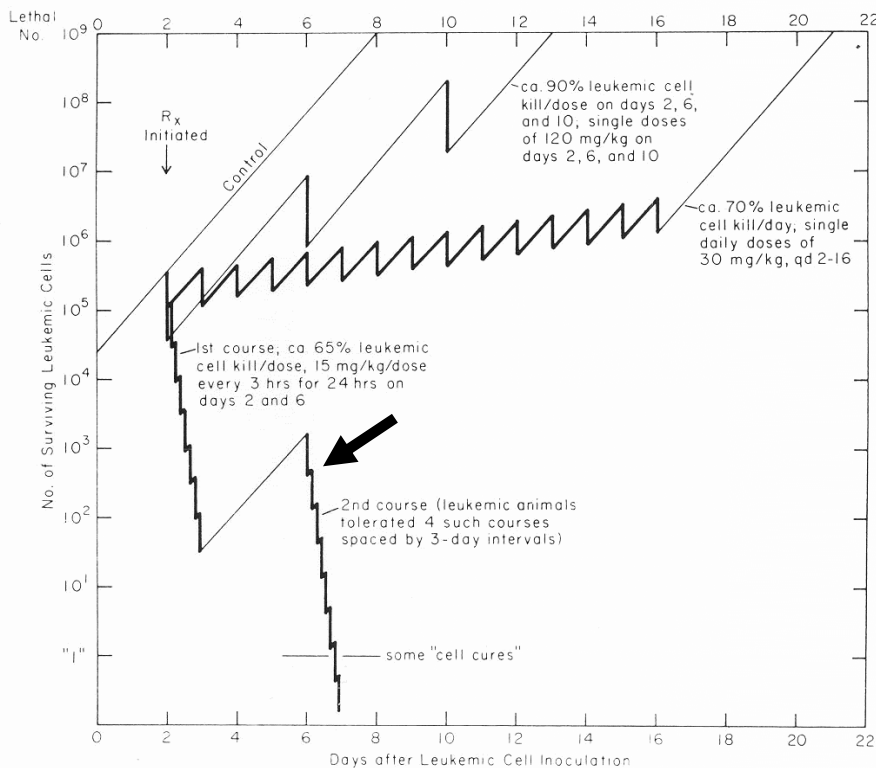


Figure 8.6. Schematic results obtained using three chemotherapy schedules of cytosine arabinoside in the L1210 system in the mouse. The effective cell kill that achieves cure is marked with an arrow. (from Skipper HE; *Perspectives in Leukemia*, NY, Grune and Stratton, pp. 187-216).

Unfortunately, many forms of acute leukemia are incurable with chemotherapy alone. The cytogenetic and molecular abnormalities found at the time of diagnosis help us to predict the likelihood of cure. If the chances of cure with chemotherapy alone are low, we may consider hematopoietic stem cell transplantation. Despite significant advances in treatment over the past 10-20 years, **acute leukemia remains a highly lethal disease**, particularly in older adults in whom the incidence of disease is highest. Five year survival rates in AML as of the year 2000

were 45% for patients younger than 45, but only 2% for patients older than 75 at the time of diagnosis. The prognosis for ALL is better, with over 80% of children and about 40% of adults achieving long-term disease-free survival.

V. Myeloproliferative Diseases

Myeloproliferative diseases are characterized by the abnormal proliferation of myeloid stem cells that retain the ability to differentiate and whose progeny function (for the most part) normally. They are categorized according to the cell type that is over-produced.

The four major myeloproliferative diseases are:

- chronic myelogenous leukemia
- polycythemia vera
- essential thrombocytosis
- primary myelofibrosis

A. Chronic Myelogenous Leukemia

Chronic myelogenous leukemia (CML) is a model malignancy. It is a clonal stem cell disorder affecting all three myeloid cell lines, most prominently the granulocytic series. It was the first neoplastic disease defined by the presence of a specific chromosomal abnormality: the Philadelphia (Ph¹) chromosome. The etiology is uncertain in most cases, but ionizing radiation or heavy benzene exposure increase the incidence of both acute and chronic myeloid leukemias in humans. CML affects a somewhat younger age group (most patients are 25 to 60 years old at the time of diagnosis) than the other myeloproliferative diseases.

The Philadelphia chromosome is formed by the reciprocal translocation of a portion of the long arm of chromosome 9 and a portion of the long arm of chromosome 22 – t(9;22) (Figure 8.7). This translocation leads to the apposition of the *c-abl* oncogene on chromosome 9 and the breakpoint-cluster region (*bcr*) of chromosome 22. *Abl* is a cytoplasmic and nuclear tyrosine kinase, and the fusion of the *c-abl* oncogene with *bcr* creates a new protein kinase. This chimeric kinase is constitutively active and has a different substrate specificity than native c-Abl, similar to that of the v-Abl gene product found in mouse leukemias induced by the Abelson leukemia virus. **Detection of the t(9;22) or the *bcr-abl* gene fusion is required to make a diagnosis of CML.** Secondary chromosomal abnormalities may appear in the abnormal clone as the disease progresses. Thus, CML is an excellent example of a malignancy in which a genetic change establishes a clone that then acquires further genetic changes, which in turn cause more aggressive behavior. As described above in the section on acute leukemia, the t(9;22) is also found in some cases of AML and ALL. In these diseases, the finding portends a poor prognosis.

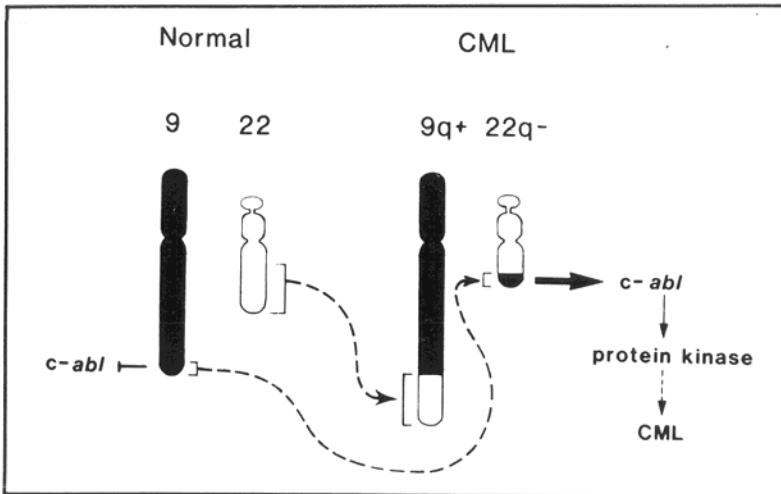


Figure 8.7. Reciprocal translocation between parts of the long arms of chromosome 9 and 22—the Philadelphia chromosome. The region on the long arm of chromosome 9 (9q), which carries the *abl* oncogene, is translocated to the distal end of chromosome 22 (22q). In its new site, the *abl* oncogene is deregulated and overproduces its protein product, the P210 protein kinase (from H. Gordon: *Oncogenes*, *Mayo Clin Proc* 60:697). Gordon: *Oncogenes*, *May Clin Proc* 60:697).

The peripheral blood in CML is a showcase of granulocytic precursor cells at all levels of development, with a differential rather like normal bone marrow. Eosinophils and basophils are numerous. The presence of basophilia and significant numbers of immature neutrophil precursors in the blood distinguish CML from reactive leukocytosis. The white cell abnormality overshadows the red cell and platelet changes; the white count may rise above 300,000/ μl in untreated patients. The platelet count is usually elevated, while the hematocrit is normal or low. This stage of the disease is called the **chronic phase**. Presenting symptoms often include fever, night sweats, fatigue (all due to hypermetabolism), and anorexia and abdominal pain caused by an enlarged spleen. Diagnosis is usually based on the peripheral blood findings described above, and cytogenetic analysis that confirms the presence of the (9;22) translocation.

CML almost always transforms to a more aggressive disease over time if not treated appropriately. Transformation is accompanied by a progressive block in differentiation. The mature WBCs in the blood and marrow are replaced by blasts, and the disease becomes acute leukemia. This is called **blast crisis** and is virtually always fatal. Interestingly, the blasts in such cases can be either myelogenous (70%) or lymphoblastic (30%).

A novel drug called **imatinib** (GleevecTM), which inhibits the new tyrosine kinase created by the *bcr-abl* gene fusion, is very effective in inducing remission and can reduce the leukemic cell burden to the point that abnormal cells can only be detected using sensitive techniques such as PCR. Patients who achieve this kind of treatment response have excellent survival and a much lower chance of developing blast crisis. However, they are not cured, and stopping imatinib usually leads to re-emergence of the disease. If an HLA-matched stem cell donor can be found, hematopoietic stem cell transplantation done during the chronic phase is often curative.

B. Polycythemia Vera

Polycythemia vera is characterized by the unrestrained, erythropoietin-independent production of red blood cells (RBC). There is often overproduction of WBCs and platelets as well. Patients with polycythemia vera typically present with symptoms related to increased red cell mass and increased blood viscosity. These symptoms may include headache, fatigue, dizziness, night sweats, visual disturbances, and weight loss. Some patients develop an unusual form of pruritus (itching) brought on by showering (aquagenic pruritus). There is an increased risk of

thromboembolic complications such as myocardial infarction, stroke, or deep venous thrombosis. There is a particularly high incidence of portal vein thrombosis. On physical examination, patients with polycythemia vera often have evidence of splenomegaly, plethora (redness) of the skin and conjunctivae, enlargement of the liver, and systolic hypertension. The spleen may become massively enlarged. The bone marrow is hypercellular, but (as is the case with all of the myeloproliferative disorders) cell differentiation is normal or nearly normal.

Our understanding of the pathogenesis of polycythemia vera and other myeloproliferative disorders has been advanced by the finding of an acquired mutation in the Janus-associated kinase 2 (JAK2) gene associated with these conditions. Encoded by a gene located on chromosome 9, JAK2 is a signal transduction protein used by the erythropoietin (EPO) receptor. Binding of EPO to its receptor causes dimerization of the receptor proteins, which in turn leads to phosphorylation of JAK2. Phosphorylated JAK2 enters the nucleus, where it generates growth and survival signals. In polycythemia vera, JAK2 is mutated so that valine is replaced by a phenylalanine at position 617. This mutated form of JAK2 (JAK2 V617F) does not require binding of EPO to the EPO receptor to become phosphorylated. This results in aberrant signaling that promotes RBC production in the absence of EPO. JAK2 signaling is also involved in regulating platelet and granulocyte production, which helps explain the high platelet and white cell counts often found in polycythemia vera. JAK2 mutations are found in 95% of cases of polycythemia vera, and at a lower frequency in essential thrombocytosis and myelofibrosis (see below).

The treatment of polycythemia vera is focused on reducing the hematocrit to levels that eliminate the risks associated with elevated blood viscosity. This can often be accomplished by phlebotomy, generally targeting a hematocrit below 45. With repeated phlebotomy, patients become iron-deficient, a desirable outcome in this situation because it limits further red cell production. Patients are usually given low dose aspirin, which reduces the incidence of thromboembolic complications. Many patients require treatment with myelosuppressive drugs such as hydroxyurea, an oral ribonucleotide reductase inhibitor. Patients with polycythemia vera can survive for many years with proper treatment, although there is an increased risk for development of bone marrow fibrosis or acute myelogenous leukemia.

DIFFERENTIAL DIAGNOSIS OF POLYCYTHEMIA

A patient with an elevated hematocrit must be evaluated for secondary causes of polycythemia. Note that **polycythemia vera** refers to the myeloproliferative disease, whereas the more general term **polycythemia** (sometimes also called **erythrocytosis**) refers to any condition in which the RBC count is elevated. Red cell production in secondary polycythemia is driven by erythropoietin (EPO). This is in contrast to polycythemia vera, in which red cell precursors proliferate in the absence of erythropoietin and erythropoietin production is suppressed. The causes of secondary polycythemia can be classified as follows:

- **Diseases that stimulate physiologic EPO production by causing chronic hypoxia at the level of the kidney.** The juxtaglomerular complex in the kidney produces EPO in response to decreased oxygen delivery to that tissue. Hypoxia-driven secondary polycythemia occurs in some patients with lung disease, chronic carbon monoxide poisoning, right to left cardiac shunts, and high affinity hemoglobins. It also occurs in healthy individuals who live at very high altitudes.

- **Diseases that cause inappropriate or ectopic EPO production.** This occurs in certain cancers, including cancer of the lung, kidney, liver, and some rare tumors of the blood vessels. In such cases, it is thought that EPO is produced by the malignant cells.
- **Administration of exogenous (pharmaceutical) EPO or a related erythropoiesis-stimulating agent** (e.g., Tour de France participants).

Secondary polycythemia can be distinguished from polycythemia vera by measuring the serum EPO level, which is typically normal or high in secondary polycythemia and very low in polycythemia vera. The presence of the JAK2 V617F mutation (see above) in a patient with polycythemia is strong evidence for the presence of polycythemia vera.

C. Essential Thrombocytosis

Essential thrombocytosis (also called essential thrombocythemia) is characterized by overproduction of megakaryocytes and platelets. There is often concomitant leukocytosis, but the hematocrit is not increased. When the platelet count is greater than 600,000/ μ l, and causes for reactive (secondary) thrombocytosis (e.g., iron deficiency, infection, or cancer) have been ruled out, the patient is considered to have essential thrombocytosis (ET). Most cases of ET defined in this way are clonal stem cell disorders in which the predominant cell line affected is the megakaryocyte/platelet lineage. The JAK2 V617F mutation is present in 50-70% of cases.

Many patients are asymptomatic, and are diagnosed with ET after an elevated platelet count is found on a routine CBC. Patients with very high platelet counts may exhibit either thrombosis or a bleeding tendency. Splenomegaly may occur, particularly in patients with the JAK2 mutation. The main goal of treatment is to reduce the risk of thrombosis and bleeding. Reduction of the platelet count with a myelosuppressive drug such as hydroxyurea decreases the risk of such complications, and most patients take low dose aspirin to prevent thrombosis. Younger patients, who have a low risk of complications, are usually monitored without treatment. As is the case with all of the myeloproliferative disorders, ET is incurable, with the exception of rare cases treated by hematopoietic stem cell transplantation. Patients with ET usually live with the disease for many years, although they have an increased risk of developing marrow fibrosis and acute myelogenous leukemia.

A patient with an elevated platelet count should undergo an evaluation to exclude secondary causes of thrombocytosis. Patients with acute hemorrhage, iron deficiency, cancer, chronic infections, chronic inflammatory disease, and those who have recently had their spleen removed all may have dramatically elevated platelet counts. Therefore, part of the evaluation of thrombocytosis should include iron studies, assessments for bleeding, and screening for cancer and infection. Bone marrow biopsy is of limited value in the differential diagnosis of thrombocytosis. The bone marrow biopsy in ET typically is somewhat hypercellular, with proliferation of megakaryocytes and increased numbers of enlarged mature megakaryocytes, all relatively nonspecific findings. If ET is suspected, molecular testing for the JAK2 V617F mutation and cytogenetic testing (to rule out occult CML, which can occasionally present with isolated thrombocytosis) are usually done.

D. Primary Myelofibrosis

Primary myelofibrosis (MF), also known as agnogenic myeloid metaplasia, is the most complex of the chronic myeloproliferative disorders. In MF, fibrous tissue infiltrates and replaces normal marrow. The fibroblasts, however, are not malignant cells. They proliferate in response to signals (growth factors) produced by neoplastic hematopoietic stem cells and their progeny. All of the chronic myeloproliferative disorders described above can produce marrow fibrosis by the same mechanism, a process that typically occurs over a period of many years. On the other hand, in primary MF marrow fibrosis is present at the time of diagnosis and is the dominant pathologic feature of the disease. Marrow replacement (myelophthisis) causes anemia and in some cases pancytopenia. Precursor cells, including nucleated red cells, giant platelets and megakaryocytes, and neutrophil precursors, appear in the peripheral blood. Red cells become misshapen (“teardrop” cells or dacryocytes) as a result of having to repeatedly traverse an enlarged spleen. Eviction of hematopoietic cells from the marrow leads to establishment of hematopoietic islands in other organs (extramedullary hematopoiesis or myeloid metaplasia), particularly the spleen and liver. Insidious weight loss, the effects of anemia, and a grossly enlarged spleen are problems that may bring the patient to medical attention. The marrow shows dense connective tissue with entrapped megakaryocytes. Almost all patients are anemic; about a third have increased platelets.

It is likely that growth-promoting factors such as platelet-derived growth factor (PDGF) released from platelets and megakaryocytes drive the fibroblasts that overrun the marrow in this disease. Studies of cell clonality using G6PD heterozygotes (see chapter 4) have shown that the hematopoietic cells, but not the fibroblasts, are derived from a single clone and, by inference, are neoplastic. The JAK2 V617F mutation is present in about 50% of MF cases. Treatment of this disease is generally unsatisfactory, and it has the worst prognosis of all the myeloproliferative disorders. Hematopoietic stem cell transplantation is the only curative option.

VI. Myelodysplastic Syndrome

Myelodysplastic syndromes (MDS) are a heterogeneous group of clonal hematopoietic stem cell disorders characterized by inability to produce adequate numbers of white cells, red cells, and/or platelets. A common feature to all forms of MDS is abnormal differentiation in the progeny of the affected stem cells, causing many of them to undergo apoptosis before reaching maturity. This often results in a state of ineffective hematopoiesis, characterized by a hypercellular marrow but decreased production of mature blood cells. MDS can result from a wide range of genetic aberrations and chromosomal losses that interfere with normal hematopoietic cell differentiation. Anything that causes genetic damage to the bone marrow can cause myelodysplastic syndrome. Two notable agents associated with the development of MDS are ionizing radiation and cytotoxic chemotherapy, and so MDS is a potential long-term complication of the treatment of other cancers. However, most cases of MDS have no apparent external cause. MDS usually evolves over a period of years, presumably as the result of accumulation of several mutations, and so patients with MDS tend to be older; the median age at diagnosis is in the range of 65 to 70 years.

The clinical and pathological manifestations of MDS are quite variable. **Almost all patients with MDS are anemic**, and many are pancytopenic. Some patients, however, have elevated white cell and/or platelet counts. Morphologic abnormalities are apparent in marrow precursors, and often in circulating blood cells. The anemia is typically macrocytic. Neutrophils may be hypolobated (so-called pseudo-Pelger-Huet cells) and/or hypogranular. Granulocytes and platelets may be dysfunctional, causing an increased risk of infection or bleeding, respectively. Marrow megakaryocytes are often abnormally small and hypolobated. Red cell precursors may exhibit megaloblastic changes (see chapter 3). Ringed sideroblasts may also be present in the marrow. Ringed sideroblasts (Fig. 8.8) are red cell precursors with coarse, iron-containing granules clustered around the nucleus, detectable only by iron stain. This is a consequence of iron accumulation in mitochondria and is an indication of disordered heme synthesis. The proportion of blast cells in the marrow is often above normal. Cytogenetic analysis often shows abnormalities involving chromosomes 5 or 7. Many cases exhibit multiple, and very complex, cytogenetic abnormalities.



Fig. 8.8. Ringed sideroblasts in a bone marrow aspirate (iron stain).

A particular concern in MDS is the tendency of the disease to devolve over time into a form of acute myelogenous leukemia. Patients with an abnormally high proportion of blast cells in the marrow (5-20%) are at highest risk for developing AML (which by definition is present when over 20% of the marrow cells are blasts). One can predict the prognosis of patients with MDS by considering the proportion of blasts in the bone marrow, the cytogenetic findings at the time of diagnosis, and the severity of the cytopenias. Not surprisingly, patients who present with a higher percent of blasts at diagnosis or who have severe, transfusion-dependent pancytopenia tend to have more rapid disease progression and shorter survival. Certain cytogenetic abnormalities, such as loss of chromosome 7, predict a high likelihood of progression to acute leukemia. In contrast, loss of the short arm of chromosome 5 predicts long survival and a good response to treatment with the drug lenalidomide. In general, however, MDS is difficult to treat and (since most patients are too old to be eligible for stem cell transplantation) incurable. Patients with low risk MDS (less severe cytopenias, no increase in marrow blasts, and no high-risk cytogenetic findings) have a median survival of 6-8 years, whereas those with high risk MDS have a median survival measured in months, not much different from metastatic lung cancer.

VII. Summary

The chapter describes a group of diseases characterized by abnormalities in the genetic control of hematopoietic cell proliferation and differentiation. Diseases characterized by increased proliferation with normal differentiation tend to be chronic diseases with a long natural history, whereas those diseases characterized by blocked differentiation cause acute illness, most notably acute leukemia. The molecular pathogenesis of some of the diseases has been elucidated, and in some cases, this knowledge has allowed the development of targeted therapy such as imatinib for the treatment of CML.

CHAPTER 9

LYMPHOCYTES AND LYMPHOMA

Key Concepts:

- Normal lymphocyte morphology, function, and development
- Pathogenesis and general classification of lymphoid malignancies
- Clinical, pathologic, and pathophysiologic features of:
 - Infectious mononucleosis
 - Hodgkin disease
 - Follicular non-Hodgkin lymphoma
 - Burkitt lymphoma
 - Chronic lymphocytic leukemia (CLL)

Learning Objectives:

Infectious mononucleosis and other benign conditions

1. Describe the pathophysiology, morphology, and laboratory and clinical manifestations of infectious mononucleosis, and distinguish it from chronic lymphocytic leukemia and other neoplastic lymphoproliferative disorders.
2. Describe the morphology of a normal (reactive) lymph node and distinguish a reactive node from lymphoma.
3. List several benign causes of lymphocytosis and lymphadenopathy.

Hodgkin disease

1. Describe the epidemiology, pathology, natural history, and clinical features of Hodgkin disease.
2. Describe the staging of Hodgkin disease and explain how the stage affects treatment.

Non Hodgkin lymphoma (NHL)

1. Compare and contrast indolent and aggressive forms of NHL in terms of epidemiology, biology, morphology, natural history, and approach to treatment.
2. Describe the role of chromosome translocations in the pathophysiology of follicular NHL and Burkitt lymphoma.

Chronic lymphocytic leukemia (CLL)/small lymphocytic lymphoma (SLL)

1. Describe the epidemiology, biology, morphology, laboratory findings, and complications of CLL/SLL.

I. Introduction

Lymphocytes, made up of T, B, and NK (natural killer) cells, account for about 1/3 of peripheral white blood cells. Most lymphocytes are found in the lymph nodes, spleen, and thymus, with substantial numbers in mucosal epithelium, skin, and bone marrow. The normal development and function of lymphocytes has been covered in other courses and therefore we will review these topics only to illustrate how developmental and activation events of lymphocytes predispose them to neoplastic transformation, and in the context of interpreting CBCs with abnormal lymphocyte numbers. We will discuss reactive and neoplastic lymphoid processes involving the blood, lymph nodes, and spleen and describe ways in which the unique biological properties of lymphocytes can assist us in distinguishing benign from malignant conditions.

II. Normal Lymphocytes

A. Numbers

About 35% of peripheral blood leukocytes are lymphocytes. The absolute normal range in adults is 1,300 to 4,200 per μl of blood. Unlike granulocytes, lymphocytes in blood do not remain constant in number throughout life. Newborns have about 5,000/ μl ; the count drops to 2,000/ μl by age ten, remains constant to age 40 and then slowly declines to 1,500/ μl by age 80. These changes are due to the loss of T cells, especially CD4 helper cells, with minor increases in B cells and cytotoxic CD8 T cells as the child matures into an adult.

An absolute lymphocyte count below 1,000/ μl is **lymphopenia**. Lymphopenia occurs in aplastic anemia, AIDS, and certain immunodeficiency diseases, after treatment with corticosteroid and other immunosuppressive drugs, and in association with some lymphomas, especially the advanced stages of Hodgkin lymphoma. The lymphocyte count is not an accurate gauge of current marrow function, however, as mature lymphocytes can proliferate outside the marrow and may remain at normal levels even in the face of recent marrow failure.

An absolute lymphocyte count above 5,000/ μl is called **lymphocytosis**. In children, lymphocytosis occurs in acute infectious diseases such as EBV-associated infectious mononucleosis and *Bordetella pertussis* infection (whooping cough). In an older adult, a persistent lymphocytosis in excess of 5,000/ μl is highly suggestive of chronic lymphocytic leukemia (discussed below).

B. Morphology and Phenotype

Traditional morphology classifies lymphocytes according to size, degree of differentiation, and according to presumptive functional characteristics (normal, atypical/reactive or plasmacytoid). These designations are useful for defining diseases. For example,

- **Small mature-appearing lymphocytes** are characteristic of chronic lymphocytic leukemia and the lymphocytosis of pertussis and other acute infections of childhood.
- **Lymphoblasts** are poorly differentiated cells found in acute lymphoblastic leukemia, the most common acute leukemia of children (Chapter 8).
- **“Atypical” (reactive) lymphocytes** are characteristic of the immune responses to infectious mononucleosis, infectious hepatitis, cytomegalovirus infections, and some

drugs. Atypical lymphocytes have a large polygonal nucleus and abundant watery-blue cytoplasm with few granules.

- **Large granular lymphocytes** (NK and T/NK cells) are also seen in viral illness, drug reactions, and autoimmune disorders. These cells have a kidney-bean-shaped nucleus and a few large red granules.
- **Plasmacytoid lymphocytes**, with features of both lymphocytes and plasma cells, synthesize IgM and are characteristic of Waldenstrom's macroglobulinemia (Chapter 10).
- **Plasma cells**, although rarely seen in peripheral blood, are easily recognized by their deep blue cytoplasm, perinuclear halo (Golgi), and eccentrically placed nucleus; they synthesize and secrete immunoglobulins IgG, IgA, IgD, or IgE. They are seen in blood during viral infections and advanced multiple myeloma (Chapter 10).

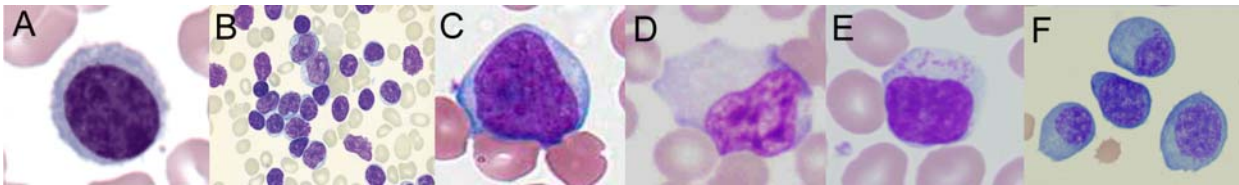


Figure 9.1. Variable morphologic appearances of lymphoid lineage cells. A) normal resting lymphocyte with dense, clumped chromatin and pale blue cytoplasm. B) Numerous small lymphocytes and prolymphocytes (with central nucleolus and increased cytoplasm) in a patient with CLL. C) A lymphoblast with fine chromatin and an easily identified nucleolus in a patient with ALL. D) A reactive lymphocyte with increased cytoplasm and enlarged nucleus in a patient with infectious mononucleosis. E) A large granular lymphocyte (LGL) with increased cytoplasm containing a small number of azurophilic granules. F) Malignant plasma cells in the pleural fluid of a patient with plasma cell myeloma (American Society of Hematology Image Bank).

Although the morphology of lymphoid cells helps to orient the physician to a diagnosis, this information is soft compared to the biologic details that can be gathered using flow cytometry (cells in suspension) and immunohistochemistry (cells in tissue sections). In both of these methods, antibodies with specific affinity for particular cellular proteins are used to identify the lineage of individual cells, either by attaching a specific fluorescent dye to the antibody, which is detectable as the cell passes through a laser light source (flow cytometry) or by attaching an enzyme (e.g., horseradish peroxidase) that can create a colored product from a specific substrate that can be viewed under the microscope (immunohistochemistry).

The CD (Clusters of Differentiation) nomenclature system was developed to create standard nomenclature for the surface proteins found on hematopoietic cells. Though it seems like a foreign language to a beginner in this field, it is an improvement from the days when CD8 was known as T8, Leu2, and Ly2, for example. For the purposes of this course, we will focus only on a few of the over 350 CD antigens that are especially important from a diagnostic, prognostic, or therapeutic standpoint. For normal mature lymphoid cells, these are listed in Table 9.1. Examples of situations in which knowledge of these CD antigens is useful in patients without any malignant lymphoproliferative disorder include assessment of total CD4 T cell numbers in an HIV infected patient, assessment for the presence of CD19 or CD20 positive B cells in a young patient being worked up for a congenital immunodeficiency, or using a monoclonal anti-

CD20 antibody (rituximab) therapeutically to eliminate autoreactive B cells in a patient with autoimmune disease.

Table 9.1

Important Protein Antigens to Distinguish Mature Normal Lymphoid Subsets

| CELL TYPE | CD MARKERS | OTHER MARKERS |
|-------------|--------------------|--|
| B Cell | CD19, CD20 | Surface Ig |
| Plasma Cell | CD138 | Cytoplasmic Ig |
| T Cells | CD2, CD3, CD5, CD7 | CD4 (Helper and Regulatory T) CD8 (Cytotoxic T) |
| NK Cells | CD2, CD16, CD56 | CD158 (Killer Inhibitory Receptors, or KIRs) |

C. Lymphoid Cell Development

Lymphoid cells, like other blood cells, are derived from hematopoietic stem cells in the bone marrow. B cells develop nearly to maturity in the bone marrow, while the earliest T cell committed progenitors exit the marrow to mature in the thymus. During the maturation process, the morphology of these progenitors ranges from similar to small lymphocytes to “blastic”, and their surface protein phenotype undergoes a number of changes. The primary event during B and T cell maturation is the rearrangement of the immunoglobulin or

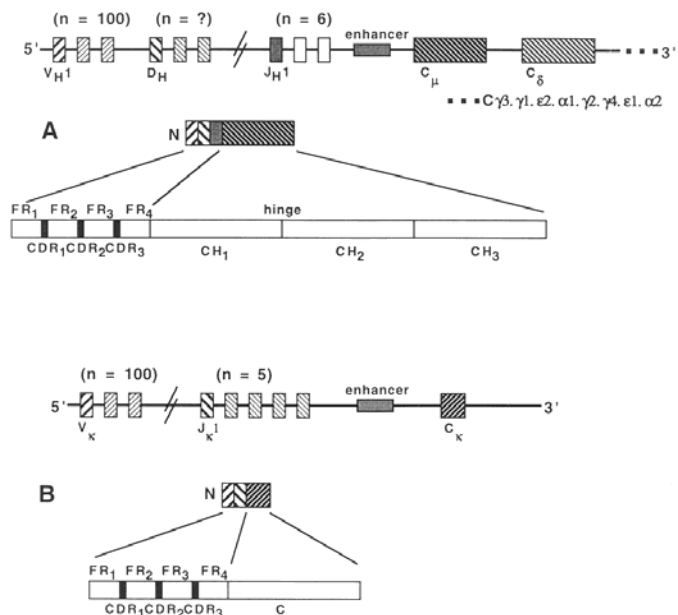


Figure 9.2. The immunoglobulin heavy chain (HC) locus (A) on chromosome 14 and kappa light chain locus (B) on chromosome 2 consist of variable (V), diversity (D), and joining (J) cassettes that are assembled into novel combinations, resulting in a vast diversity of specificities. The TCR locus is similarly organized with the α chain similar to the κ locus and β chain similar to Ig HC.

T cell receptor chain genes (Figure 9.2). This generates cells able to produce proteins that can recognize a nearly infinite set of potential foreign antigens. Development of frankly self-reactive B cells or T cells, on the other hand, is suppressed.

These gene rearrangements require producing double-stranded breaks in chromosomes and rearranging the genome of T cells and B cells, a “risky business” that must have evolved under intense pressure to create an adaptive immune system. As you might guess, this process is not error-proof, and can occasionally result in placing powerful, lineage-specific promoters, such as those that drive TCR and Ig gene expression, in front of oncogenes such as *c-myc* or *bcl-2*. This causes unregulated oncogene overexpression, and in combination with other genetic events can result in lymphoma, as discussed below.

If they are successful in producing a functional TCR or Ig molecule, T and B cells migrate to secondary lymphoid organs (lymph nodes and spleen), where they become fully mature. Naïve B cells aggregate in primary follicles, arrayed around the cortex of the lymph node, while T cells take up residence between these follicles (perifollicular region) and extend down into the medulla, an area with prominent vascular/lymphatic sinus structures containing scattered plasma cells and macrophages (Figure 9.3). A key cell in lymph node biology is the follicular dendritic cell (FDC), an antigen presenting cell that generates a web of dendritic processes in the B cell follicle. These FDC can be identified by staining for CD21 or CD23.

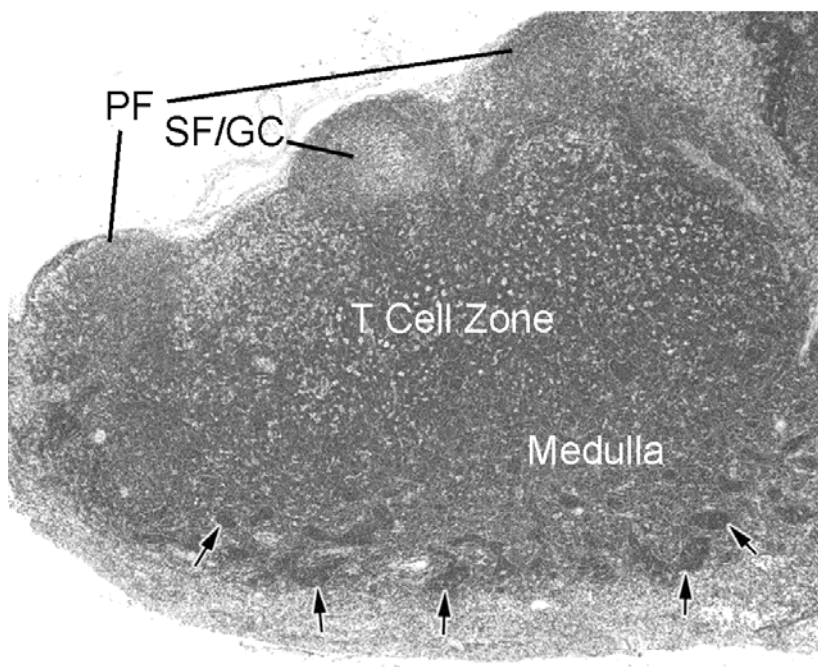


Figure 9.3. Normal Lymph Node. In the cortex at the top and left of image, primary follicles (PF) containing resting naïve B cells, as well as a small secondary follicle (germinal center, SF/GC) containing B and T cells reacting to an antigen are present. Both types of follicles are organized by networks of FDC. The moth eaten look of the T cell paracortical zone is due to scattered histiocytes (tissue macrophages) and larger activated lymphoid cells (immunoblasts) in the midst of smaller naïve T cells and memory cells. In the medulla, lymphatic sinusoids are stuffed with migrating small lymphocytes (arrows). Image modified from: Cynthia L. Willard-Mack *Toxicologic Pathology*, 2008.

If a B cell encounters an antigen that binds to its cell surface Ig molecule and causes crosslinking of these molecules, the B cell becomes activated. For most antigens, the presence of T cells that also recognize peptides from the antigen presented on FDC and B cells in the vicinity is required for the B cells to become fully activated, proliferate, produce costimulatory molecules that further activate T cells, and secrete cytokines. During this process, two other events that affect the genome and are potential contributors to lymphomagenesis occur: immunoglobulin class switching and somatic hypermutation (Figure 9.4). As B cells become “eligible” for T cell help in the germinal center by virtue of their ability to bind protein antigens with their surface Ig

molecules, they undergo proliferation and hypermutate the VDJ and VJ (light chain) regions of the heavy and light chain genes that encode the sequence responsible for antigen binding. While this random process likely results in decreased affinity in most cases, in some cells the affinity for antigen will be increased, enhancing the ability of this particular B cell to bind antigen and present the peptides of this antigen to T cells. This is an iterative process that generates B cells producing very “good” (high-affinity) antibodies in large amounts, and eliminates cells that make less effective antibodies. Although the somatic hypermutation process is directed at specific regions of the Ig gene, errors can occur, causing mutations in oncogenes such as BCL6 or MYC and abetting

lymphomagenesis. B cells must undergo another round of double stranded chromosome breakage, with the attendant risk of a harmful mutation, when they undergo immunoglobulin class switching (switching from making IgM/IgD to making IgG, IgA, or IgE), a process that requires placing the same variable portion of the Ig gene onto a different constant tail.

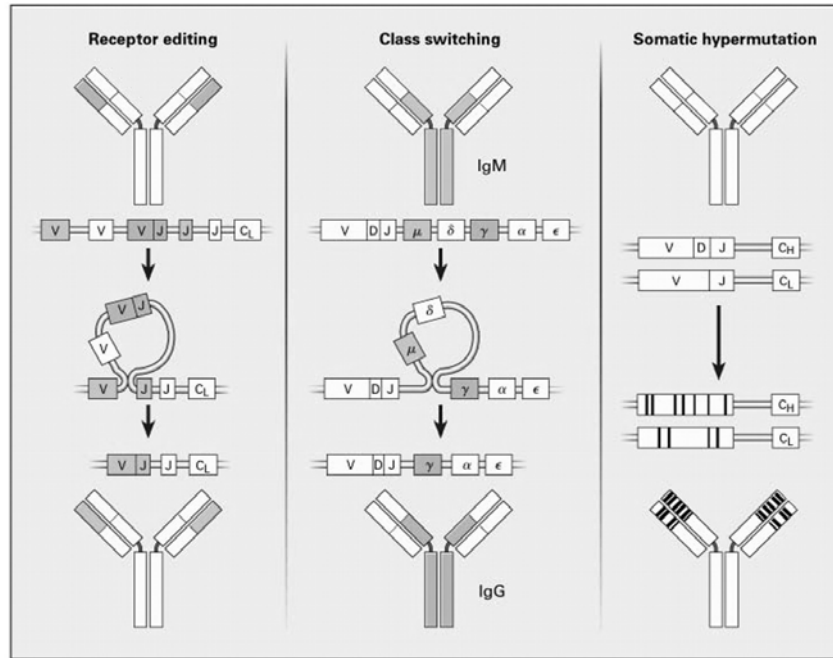


Figure 9.4. Murphy et al. *Janeway's Immunobiology*, 7th Ed. Developing B cells that find themselves binding self antigens in the bone marrow can ‘edit’ their light chain by redoing the rearrangement to more distal V (variable) and J (joining) cassettes in an attempt to produce a non-self-reactive immunoglobulin (left). In the germinal center, B cells recognizing protein antigen are induced to undergo both somatic hypermutation of their Ig genes in the areas encoding the antigen binding region (right) and to isotype switch from IgM/IgD production to IgG, IgA, or IgE, etc. The same VDJ region is maintained, so antigen specificity is the same while other features such as complement binding, ability to cross the placenta or mucosal surfaces, etc. is altered (center). All of these events can be associated with lymphoma-inducing translocations and mutations.

III Lymphoid Malignancy

Lymphomas are solid tumors consisting of lymphoid cells. They result from clonal expansion of lymphocytes of B, T, or NK origin and usually arise in lymphoid tissue. Lymphomas are often recognized clinically by enlargement of peripheral lymph nodes. Extranodal lymphomas arise from lymphocytes present in other organs.

For classification purposes, malignant lymphoma is divided into two main categories: Hodgkin lymphoma and non-Hodgkin lymphoma. Non-Hodgkin lymphomas (NHL) are about six times more common than Hodgkin lymphoma (HL). In both groups there is a male predominance. For Hodgkin lymphoma, there is a characteristic bimodal age/incidence curve, with 50% of the cases of Hodgkin lymphoma occurring between the ages of 20 and 40, and a second peak between the ages of 60 and 80. The peak incidence period for non-Hodgkin lymphomas is later, with a median age of about 60.

All types of lymphoma may present with painless swelling of the lymph nodes. Any group of nodes may be involved, but the cervical nodes are the most frequently involved. Enlarged lymph nodes are especially common in children as a response to infection, but a persistent posterior cervical, supraclavicular, or axillary node measuring >2x2cm despite treatment with antibiotics is a clue to malignancy.

A surgical biopsy is required to establish the diagnosis. The specimen is submitted for histology and for immunohistochemistry or flow cytometry analysis (to determine immunophenotype) and occasionally molecular studies. Lymph node pathology is a challenging discipline, as there are 60 distinct lymphoma subtypes in the most recent World Health Organization (WHO) classification, and distinguishing one subtype from another requires an expert lymph node pathologist, able to integrate histological, immunophenotypic, and molecular information. In addition, benign conditions such as EBV infection or reactive follicular hyperplasia can resemble lymphoma histologically.

A. Hodgkin Lymphoma

HL is a lymphoma characterized by the presence of Reed-Sternberg (RS) cells in the biopsy. HL is relatively common in young adults and many cases are linked to EBV infection. One confusing aspect of Hodgkin lymphoma is that it is divided into “classic” HL and another malignancy called “nodular lymphocyte predominant HL.” The latter condition is really better thought of as a large B cell lymphoma with a prominent infiltrate of reactive B and T cells and carries a very favorable prognosis. In this course we will consider only classic HL.

Hodgkin lymphoma differs from the non-Hodgkin lymphomas in the following respects:

- It is **curable** in about 70-80% of patients (significantly higher cure rate than in NHL).
- It is believed to begin in a single lymph node and then spread via lymphatic channels to adjacent nodes (NHL more likely to spread through the blood and involve many distant nodes simultaneously).
- The malignant cell, the unique Reed-Sternberg cell, makes up a minority of the “tumor” but is a crucial component of the pathology.
- There is a polycellular response of normal neutrophils, eosinophils, lymphocytes, and plasma cells and fibroblasts in the involved tissue.
- T cell immune function is poor in Hodgkin lymphoma, whereas humoral immunity is more likely to be deficient in the B cell and T cell lymphomas.

1. Pathogenesis

The malignant cell in HL is the Reed-Sternberg (RS) cell. RS cells are large cells typically with bilobed nuclei. They are derived from B lymphocytes that have gone through a germinal center reaction, but have lost the ability to make functional immunoglobulin. **RS cells are potent cytokine producing cells that generate an inflammatory reaction** of neutrophils, eosinophils, and plasma cells. The etiology is unknown, although EBV is found in the tissue in 50% of cases. It is not known whether EBV is a passenger virus or important in the pathophysiology.

2. Immune dysfunction

Abnormalities in cellular immunity such as skin test anergy and decreased levels of circulating T cells are found in patients with Hodgkin lymphoma. These defects are more frequent in patients with advanced disease, and may persist after treatment. Consequently, patients with Hodgkin lymphoma are more susceptible to fungal and viral infections, such as cryptococcosis and herpes zoster. Immunoglobulin production is preserved.

3. Pathology

All cases have diagnostic RS cells in the presence of a background of small lymphocytes, eosinophils, and plasma cells. In tissue sections, the RS cell is a large binucleate or multinucleate cell with a large eosinophilic nucleolus in each nucleus. These features often give the cell an “owl-eyed” appearance (Figure 9.5). Although RS cells are derived from B cells, their immunophenotype is as aberrant as their appearance. Unlike most B cells, they usually lack CD20, CD19, and CD45, but express CD30. The presence of a prominent background of benign cells of mixed lineage is an important diagnostic feature. In many cases, this mixed infiltrate is accompanied by prominent fibrotic bands and sclerosis of the lymph node capsule (a common variant called nodular sclerosing HL).

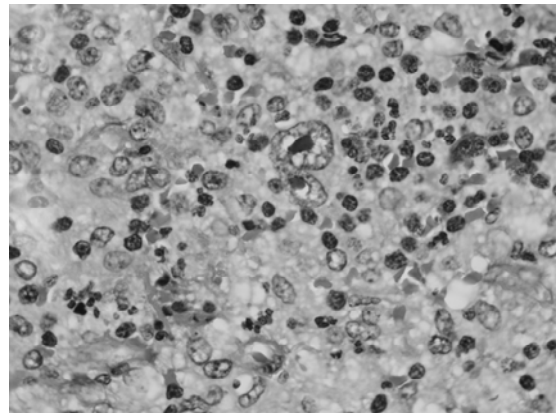


Figure 9.5. A binucleate Reed-Sternberg cell with large nucleoli is surrounded by a mixed infiltrate of lymphocytes, eosinophils, neutrophils, and plasma cells in this case of Classic Hodgkin Lymphoma.

4. Clinical Features

HL can present at any age but is most common in young adults and the elderly. Most patients seek medical attention after discovering painless lymphadenopathy, often in the neck or supraclavicular fossa. About 25% of patients will present with symptoms such as cough (due to mediastinal adenopathy) or constitutional (“B”) symptoms. B symptoms (so-named because of their role in the Ann Arbor staging system described below) are defined as recurrent fever, recurrent drenching night sweats, or unexplained loss of 10% body weight in a six-month period. Pruritis (which is not a B symptom) is also seen in a minority of patients. Common laboratory abnormalities include normocytic anemia, lymphopenia, an

elevated erythrocyte sedimentation rate (ESR), and an elevated lactate dehydrogenase (LDH) level.

Staging of all lymphomas is done according to the Ann Arbor staging system (Fig. 9.6).

- Stage I indicates node involvement on one lymph node area.
- Stage II indicates disease involving two or more nodal areas confined to one side of the diaphragm.
- Stage III indicates nodal disease above and below the diaphragm.
- Stage IV indicates widespread involvement including disease outside the lymph node system.

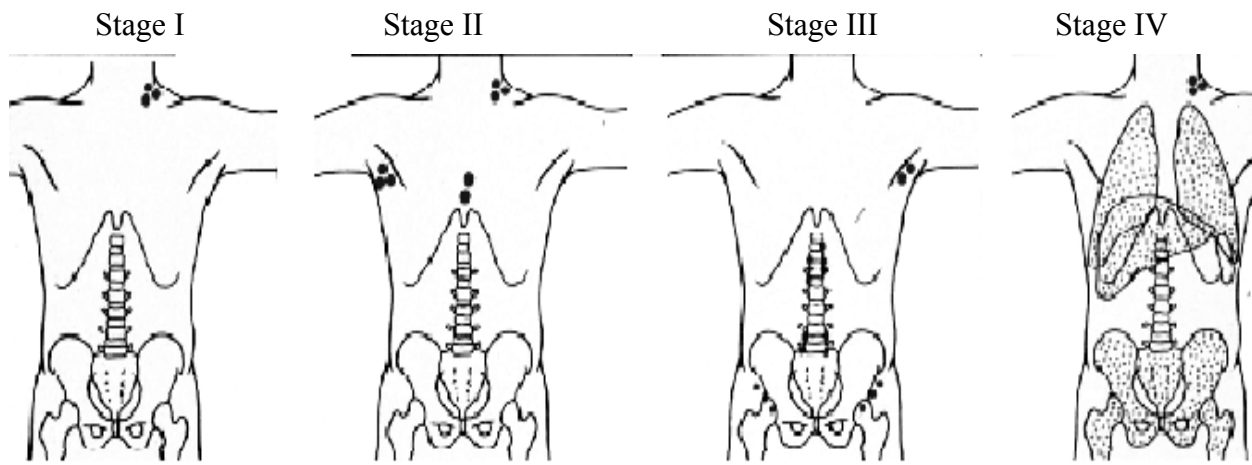


Figure 9.6. Ann Arbor staging of HL is determined by the extent and location of involved lymph nodes (or spleen) and in the case of Stage IV disease, the marrow or other non-lymphoid tissues. Adapted from Skarin, *Dana-Farber Cancer Institute Atlas of Diagnostic Oncology*, 1991.

The stage of the disease has an important influence on prognosis. Stages of Hodgkin lymphoma (I-IV) are further divided into “A” or “B” (i.e., IA, IB, IIA, IIB, etc), depending on whether or not the constitutional symptoms listed above are present. “A” indicates the absence of such symptoms.

Since Hodgkin lymphoma spreads in a nonrandom fashion, and since treatment is designed according to extent of disease, it is most important to know the extent of disease (i.e., the stage) prior to treatment. Staging for Hodgkin lymphoma usually includes computerized axial tomography (CT) of the chest, abdomen, and pelvis (and often neck), and bone marrow biopsy. Positron emission tomography (PET scanning), which can identify areas of malignant spread based upon associated increased metabolic activity, is a useful staging tool in many cases.

5. Treatment

Treatment for Hodgkin lymphoma depends on the stage of the disease. Abbreviated chemotherapy plus radiotherapy is the main mode of therapy for patients in Stage I and II. Radiotherapy is delivered to known areas of disease and immediately adjacent node-bearing tissue, taking into account the tendency for contiguous spread of the disease. More advanced disease is treated with repeated cycles of combination chemotherapy.

The prognosis for Hodgkin lymphoma has greatly improved with modern therapy.

The prognosis for five-year survival for

- Stage I and II disease is 85% to 95%;
- Stage III, 70% to 85%;
- Stage IV 55 to 70%.

Children do as well as adults, although the disease is rare under 10 years of age. With the improvement in prognosis, less aggressive treatment regimens are now being studied in an attempt to decrease the amount of therapy given to patients with favorable prognosis and thereby avoid some of the long-term side effects of therapy (e.g., sterility, growth abnormalities in children and other organ dysfunction from radiation therapy, and most critically, secondary malignancies such as sarcoma and acute leukemia).

B. Non-Hodgkin Lymphoma (NHL)

1. Classification

The classification of lymphoma has changed over the years with a better understanding of the underlying biology of the disease. Lymphoma cases are now classified according to cell of origin (B, T or rarely NK cell), stage of maturation (precursor or mature cells), histology, and underlying genetics. The majority of lymphoma cases in the US (80%) are B-cell lymphomas.

Non-Hodgkin lymphomas fall into two broad categories: indolent and aggressive.

- **Indolent (low grade) lymphomas** are generally slower-growing, composed of more mature cells, and, although they are often treatment-responsive, incurable. They affect mainly older individuals. Some forms of low grade lymphoma exhibit such indolent behavior that patients are not typically treated unless the disease is causing symptoms.
- **Aggressive (high grade) lymphomas** are faster-growing and composed of less mature cells. They generally exhibit relentless (and in some cases very rapid) progression and are fatal if untreated. However, in contrast to low grade lymphomas, many patients can be cured with treatment. Most high grade lymphomas occur in middle age or later, but some (Burkitt lymphoma, for example) occur with appreciable frequency in children and young adults.

According to the most recent World Health Organization (WHO) classification scheme, there are over 60 different kinds of lymphoma. Because lymphocytes go through many stages of differentiation on their way to becoming a fully mature cell, there are many “opportunities” for different lymphomas to develop. **The most common indolent lymphoma is follicular lymphoma (a B-cell lymphoma) and the most common aggressive lymphoma is diffuse large B cell lymphoma. Together, these account for about 50% of non-Hodgkin lymphomas.**

2. Pathogenesis

When a B cell passes through the germinal center of a lymph node and undergoes somatic hypermutation and class switching, it is particularly vulnerable to malignant transformation. Many lymphomas are the result of chromosomal translocations involving various oncogenes and the promoter for the IgH gene on chromosome 14.

Table 9.2

COMMON TRANSLOCATIONS IN LYMPHOMAS

| <u>Tumor</u> | <u>Translocation</u> | <u>Comment</u> |
|-----------------------|-----------------------------|---------------------------|
| Follicular lymphoma | t(14;18): IgH + Bcl-2 | Decreased apoptosis |
| Large cell lymphoma | t(3;14): IgH + Bcl-6 | Deregulated transcription |
| Plasmacytoid lymphoma | t(9;14): IgH + PAX-5 | Unknown effect |
| Mantle cell lymphoma | t(11;14): IgH + Cyclin D | Increased proliferation |
| Burkitt lymphoma | t(8;14): IgH + c-myc | Increased proliferation |

The t(14;18), which is found in almost all cases of follicular lymphoma, places the *bcl-2* oncogene (chromosome 18) under the control of the IgH promoter (chromosome 14), leading to over-expression of the Bcl-2 protein. Bcl-2 expression increases the cell’s apoptotic threshold (in other words, sends a “don’t die” signal). The result is a rather indolent lymphoma characterized by slow accumulation of malignant cells in lymph nodes resulting in lymph node enlargement.

The t(8;14) that characterizes Burkitt lymphoma places the *c-myc* oncogene (chromosome 8) under the control of the IgH promoter (Figure 9.7). Overexpression of *c-myc* creates a strong proliferation signal and so Burkitt lymphoma is a very aggressive malignancy with an extraordinary growth rate (tumor nodules may double in size in one day).

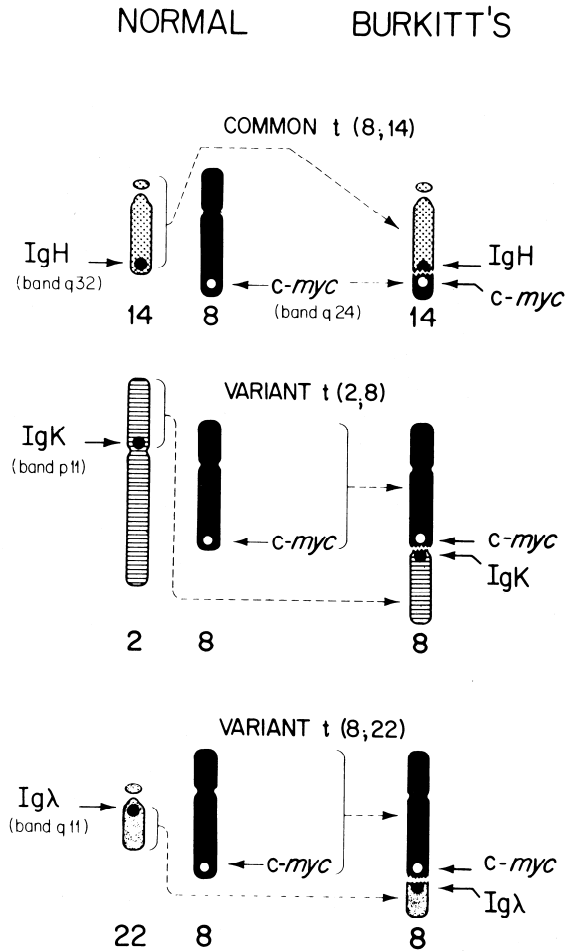


Figure 9.7. The common t(8;14) and variant t(2;8) and t(8;22) chromosomes in Burkitt lymphoma. *c-myc* is indicated in white dots and the immunoglobulin genes in black dots. The normal chromosomes are illustrated on the left and the abnormal *c-myc*-bearing chromosomes on the right. The second abnormal chromosome produced by each translocation (which may contain nonfunctional fragments of *c-myc* or immunoglobulin genes) is not represented, nor is the normal chromosome of each pair, which does not participate in the translocation. (From Aisenberg AC. New genetics of Burkitt lymphoma and other non-Hodgkin lymphomas, *Am J Med* 77:1083).

Other factors that may contribute to the pathogenesis of lymphomas in humans include chronic infection and immunosuppression. These factors may be independent or may act in concert. Examples of infections that appear to increase lymphoma risk include EBV, hepatitis C, and *H. pylori*. Examples of immunosuppressed patients with an increased risk of lymphoma include patients with AIDS and organ transplant recipients who are given immunosuppressive drugs to prevent allograft rejection. Most post-transplant lymphoproliferative disorders (PTLDs) are due to a combination of immunosuppression and EBV infection.

3. Clinical presentation

As with Hodgkin lymphoma, the most common presentation of NHL is palpable lymphadenopathy. Individuals may also present with B symptoms, pain, organ failure, or a variety of other symptoms, depending upon the kind of lymphoma and the sites of involvement. Patients with indolent lymphoma are more likely to be asymptomatic at diagnosis than patients with aggressive lymphomas. More than two thirds of patients with B-cell lymphoma have disseminated disease at diagnosis. Many have extranodal disease, especially in the bone marrow and gastrointestinal tract. Some patients have circulating lymphoma cells.

Immunologic abnormalities may be found in association with B-cell lymphomas, especially in chronic lymphocyte leukemia/small lymphocytic lymphoma (discussed below). These abnormalities include Coombs-positive hemolytic anemia, immunoglobulin deficiency and monoclonal gammopathy. Patients with NHL are more prone to bacterial than fungal or viral infections, in contrast to patients with Hodgkin disease.

4. Diagnosis and staging

An adequate sample of tissue is essential to establish the correct diagnosis.

Distinguishing one lymphoma subtype from another requires a skilled pathologist using a combination of morphology, immunophenotyping, and occasionally molecular diagnostics. Fine needle aspiration may help distinguish lymph nodes enlarged by lymphoma versus metastatic carcinoma or reactive processes, but accurate subtyping and characterization of lymphomas requires excisional biopsy.

FYI

As mentioned above, most NHL are of B cell origin. Within this broad category, subclassification of the different types of B cell lymphoma relies on the morphology, architectural organization, immunophenotype, and in some cases, the specific genetic aberrations of the malignant cells (e.g., the MYC translocation in Burkitt's lymphoma or the characteristic t(11;14) translocation with overexpression of Cyclin D1 in mantle cell lymphoma). Previous pathologic classification schemes relied primarily on the size and shape of the malignant cells and the architecture of the infiltrate (e.g., "diffuse, small cleaved cell lymphoma"). The present-day WHO classification system makes a greater effort to incorporate biologic and clinical information into the scheme to make it more biologically and therapeutically relevant. Some B cell lymphomas are named for the resemblance between the malignant clonal cells and a normal counterpart (geographic locale in the lymph node, immunophenotype, or morphology), as in mantle cell lymphoma and marginal zone lymphoma. Others still rely on morphology and architecture, as in diffuse large B cell lymphoma (DLBCL). In this case, diffuse effacement of normal lymph node (or other tissue) by large atypical cells that immunophenotypically are identifiable as B cells (CD20⁺ in most cases, though other B cell markers such as CD79a, PAX5, and CD19 can also be used) is diagnostic of DLBCL.

Generally speaking, the hematopathologist is assessing whether there are significant abnormalities in lymph node architecture (loss of the typical B and T cell zone pattern), aberrant locations or accumulations of B cells or T cells, or the presence of cells with atypical morphology (e.g., large cells or RS cells) in order to render a diagnosis of lymphoma. In some cases, it can be difficult to distinguish reactive and malignant conditions. In these situations, flow cytometric analysis or genetic analysis by PCR (or Southern blot) can be performed to determine whether a clonal immunoglobulin (or TCR for T cell lymphoma) gene rearrangement is present. As this rearrangement and use of specific V, D, and J cassettes is unique for every lymphocyte, a malignant lymphocyte population will show an aberrant monoclonality in this regard. This is a unique tool to assess malignancy that is available in lymphoid proliferations but lacking in other cancers (other cancers clearly have clonal genetic aberrations, but we do not know up front where to look, unlike in lymphoid lesions that always have clonal Ig or TCR gene rearrangements).

Staging of NHL patients includes a history and physical, routine lab work including beta 2 microglobulin (which has prognostic relevance in indolent lymphomas), and LDH (which has prognostic significance in aggressive lymphomas). CT scans of chest/abdomen/pelvis and a bone marrow biopsy are used to determine the extent of disease. PET scanning can provide additional information beyond the CT scan, particularly in metabolically active (i.e., aggressive) lymphomas. Finally, some patients with aggressive lymphomas require evaluation of the central nervous system with a lumbar puncture and CSF examination.

The Ann Arbor staging system described above is also used for NHL. **Stage has a more important role in determining prognosis and treatment in Hodgkin lymphoma than in non-Hodgkin lymphoma. In NHL, the histologic subtype is the most important factor in determining the prognosis and treatment.**

5. Treatment

Therapy is individualized according to the patient's age and the natural history of the specific lymphoma. In some forms of indolent lymphoma, therapy can be postponed until the patient becomes symptomatic. As most NHL are almost by definition “metastatic” at presentation and not amenable to surgical excision, early detection of indolent lymphoma is less critical than it is, for example, in breast cancer or colon cancer. For aggressive lymphomas, combinations of drugs that include agents such as anti-CD20 (rituximab – useful in B-cell lymphomas that express CD20), cyclophosphamide, vincristine, adriamycin, and prednisone can induce remission in most patients and a majority of patients can be cured. Radiotherapy, either alone or in combination with chemotherapy, may help to palliate localized symptomatic disease. **In general, the goal of treatment in patients with indolent NHL is control of symptoms and improvement in quality of life, while the goal of treatment in a newly-diagnosed patient with aggressive lymphoma is cure of the disease (assuming the patient is healthy enough to tolerate intensive chemotherapy).**

Non-Hodgkin lymphomas in children are typically aggressive cancers that have many features in common with acute lymphoblastic leukemia (discussed in chapter 8). At diagnosis, children are more likely than adults to have disseminated disease. An aggressive approach to the therapy of high grade lymphomas in children, using central nervous system treatment along with combination chemotherapy, has resulted in a remarkable improvement in the survival.

Prognosis depends on age (older patients have a worse prognosis), pathologic classification, immunologic function, stage and mass of disease, growth rate, site (brain lymphoma has a very bad prognosis), lactic dehydrogenase level (LDH, a marker for high cell turnover), symptoms, and performance status (a measure of the patient's overall health and ability to do regular activities).

IV. Specific Diseases and Their Diagnosis

In this final section, we will discuss a few representative lymphoid malignancies, and describe their presenting features and diagnostic strategies that separate them from benign/reactive conditions. For a much more detailed discussion of specific lymphomas the student is directed to the *WHO Classification of Tumors of the Haematopoietic and Lymphoid System*, (4th ed., 2008).

A. Lymphocytoses – Reactive vs. Neoplastic

In Chapters 1 and 8, we addressed the approach to high and low peripheral blood counts in the myeloid and erythroid lineages. When a patient presents with a high absolute lymphocyte count (>3-4K/ μ l) on the CBC, one of the key questions to ask is also the simplest, “How old is the patient?” **In a person under age 40, lymphocytosis is most often reactive (Table 9.3). One of the more common causes of reactive lymphocytosis conditions is acute infection with Epstein-Barr virus (infectious mononucleosis).** Infectious mononucleosis presents with impressive symptoms and signs in young adults: fever, sore throat, profound fatigue, enlarged lymph nodes in the neck and axillae, rash, splenomegaly and, less often, hepatomegaly. Symptoms usually last one to four weeks. Some of these clinical features also may be found in patients with cytomegalovirus and toxoplasma infections. More complicated manifestations such as splenic rupture, aseptic meningitis, respiratory distress as a result of enlarged tonsils, and severe hepatitis can be seen but are rare. There is no specific treatment.

Table 9.3 – Causes of Lymphocytosis

| Infection | Drug | Other | Endocrine | Malignant |
|---|----------|---|--|--|
| EBV, CMV, Hantavirus, early HIV, brucellosis, toxoplasmosis, TB, pertussis (can be dramatic in children), other | Dilantin | Smoking – polyclonal B cell lymphocytosis Post-vaccination | Thyrotoxicosis Acute stress (transient) | CLL , large granular lymphocytic leukemia, peripheralization of other lymphoid malignancies including mantle cell, marginal zone, and follicular lymphoma |

There are many hematologic changes associated with infectious mononucleosis. Lymphocytosis with a preponderance of atypical lymphocytes is most frequently seen several days into the clinical illness. The “atypical” lymphocytes are larger than normal, with increased cytoplasm that tends to mold around neighboring RBC (see Fig. 9.1.d). Despite the fact that EBV preferentially infects B cells, the circulating atypical lymphocytes are mainly CD8+ T cells that react specifically to EBV-produced antigens. Anemia is rare. A mild reduction in platelet number is found in 25% of cases. Elevation in liver enzymes is found in 80% of the cases, indicating mild hepatitis.

In order to help confirm the diagnosis of infectious mononucleosis, a rapid but not very specific test (**Monospot**) can be performed. This test assesses for the presence of so-called **heterophile antibodies**, non-specific antibodies released in increased amounts by activated B cells that cause agglutination of horse RBC or hemolysis of ox RBC. Specific tests for infectious mononucleosis involve assaying for antibodies against components of the EB virus. Early in the infection there is a rise in IgM antibodies against viral capsid antibody (VCA) and antibodies against the so-called "early antigen" (EA). Later, the IgG VCA appears; this persists for life. Antibody against Epstein-Barr nuclear antigen (EBNA) develops after several weeks. Quantitative PCR assays that enumerate EB virions in the blood are also available.

B. Malignant Lymphocytoses

In older patients, malignancy is the most common cause of peripheral lymphocytosis, especially chronic lymphocytic leukemia (CLL). CLL is the most common leukemia in adults (at least in Western countries; it is quite rare in Japan). Symptoms are often minimal at the outset, so that CLL is often diagnosed incidentally when the patient is being seen for some other problem and is found to have a high lymphocyte count on the CBC. 90% of CLL patients are over 50, and the incidence reaches 25 per 100,000 at age 70. There is a genetic predisposition in some families.

1. Diagnosis of CLL

Peripheral blood smears show large numbers of normal-appearing small lymphocytes, from 5000 to >500,000/ μ l. "Smudge cells" (cells that have disintegrated during the preparation of the blood smear) are often prominent. CLL cells have a characteristic immunophenotype. They are monoclonal B-cells that express monoclonal IgM + IgD (all cells express either κ or λ light chains) on their membranes as well as CD5 (normally a T cell antigen), CD19, low amounts of CD20, and CD23.

FYI

Recent experimental work has revealed that CLL likely includes two distinct disease processes with different prognoses. In one "flavor" of CLL, the monoclonal B cells exhibit somatic hypermutation in the Ig variable genes, indicating that they have passed through the germinal center. These patients have a good prognosis, with a median survival of over 10 years. In contrast, patients whose malignant clone shows little or no evidence of somatic hypermutation, presumably reflecting origin from a naïve B cell, have a worse prognosis, with median survival in the 5-7 year range. In this latter group the leukemic cells typically express CD38 and the T cell signaling molecule ZAP-70.

Flow cytometry is usually employed to diagnose CLL or other disorders that present with peripheral lymphocytosis. The flow cytometer used for these analyses is similar to that used in automated CBC analyses, but is also equipped to detect the level of fluorescence emitted from the dye-tagged antibodies binding to each cell as it passes through the laser. By staining the blood, bone marrow, or lymph node sample with cocktails of antibodies against specific surface proteins that have different fluorescent dyes attached, the flow

cytometer can detect even relatively small clones with aberrant phenotypes. It can also determine whether the normal ratio of kappa vs. lambda expressing B cells (2-3:1) is significantly altered. Since each B cell “chooses” to express either kappa or lambda light chain, but not both, during development, all of that cell’s progeny will also express the same light chain. Thus, in the case of CLL or other B cell neoplasm the presence of large numbers of malignant cells will skew the $\kappa:\lambda$ ratio heavily in one direction or the other. For the diagnosis of CLL specifically, we also look for co-expression of CD19 with CD5 on the same cell (suggests CLL or mantle cell lymphoma) and for expression of CD23 (positive in CLL, negative in mantle cell lymphoma). Other B cell malignancies have other unique phenotypic characteristics.

It can be confusing that in a disease we call chronic lymphocytic **leukemia**, patients very often exhibit lymphadenopathy and splenomegaly due to infiltration by leukemia cells. Indeed, in patients where these features are more prominent and the peripheral blood and bone marrow involvement is less obvious, the term small lymphocytic lymphoma (SLL) is used. For the purposes of this course, **SLL and CLL can be considered synonymous**, though presumably there are biologic differences between the clonal cells in patients that present in a more “CLL-like” way vs. those who are more “SLL-like”.

FYI

In both cases, the lymph node architecture is effaced by uniform, small lymphocytes. Germinal centers are absent, but paler-staining, irregular patches containing somewhat larger proliferating CLL B cells, T cells, and dendritic cells can be seen. These are called proliferation centers or “pseudo-follicles” and are not true germinal centers; they lack tingible body macrophages and clear, dense dendritic cell networks. In situations where fresh, disaggregated tumor cells are not available for flow cytometric analysis, expression of CLL-specific antigens can be assessed by immunohistochemistry on tissue sections.

2. Clinical Features of CLL

One of the most striking and paradoxical clinical features in CLL is the combination of immunodeficiency and a propensity towards autoantibody-mediated immune phenomena, particularly autoimmune hemolytic anemia and immune thrombocytopenia (ITP) that is seen in these patients. Given the indolent nature and slow growth of the malignant B cells, it is perhaps not surprising that many CLL patients die from infection or as the result of cytopenias, rather than from organ dysfunction mediated by infiltration of malignant B cells *per se*.

Two factors contribute to the immunodeficiency state of CLL patients:

- Decreased levels of normal immunoglobulins (seen in at least 50% of patients)
- T cell deficiency, evidenced by anergic DTH responses to skin tests, poor responses to vaccination, and increased incidences of both bacterial and viral infections, including shingles (varicella-zoster virus reactivation). Malignant B cells in CLL secrete immunosuppressive cytokines (TGF- β) and soluble forms of cell surface receptors such as CD27 that inhibit T cell function. Another manifestation of this immunodeficiency is the increased incidence of second malignancies, such as carcinoma of bowel, lung, skin, prostate, and bladder that is noted in CLL patients.

Autoimmune phenomena in CLL are a manifestation of dysregulation of the immune system, with resultant loss of tolerance to self-antigens. In some cases the autoantibodies are produced by the leukemic cells themselves, but more often they are produced by “normal” (non-neoplastic) B-cells.

As is the case with other indolent lymphomas (particularly follicular center cell lymphoma), in a minority of cases transformation to a much more aggressive (and rarely curable) large cell lymphoma may occur. In the case of CLL, this is called “Richter’s transformation”.

CLL patients who are asymptomatic generally do not require treatment. Patients who are symptomatic or who have rapidly progressive disease often benefit from treatment with chemotherapy with or without rituximab (anti-CD20 mAb), but the disease remains incurable except in some cases following allogeneic bone marrow transplant.

FYI

Other Malignant Lymphocytoses

A detailed review of all such diseases is beyond the scope of our discussion, but less common neoplasms presenting as lymphocytoses include large granular lymphocytic leukemia, a proliferation of NK or T/NK cells that is often associated with autoimmune blood disorders such as red cell aplasia or neutropenia, or peripheral blood involvement by lymphomas that more typically reside in lymph nodes or bone marrow, such as mantle cell, splenic marginal zone, or follicular lymphoma. These can often be distinguished by a combination of clinical history, flow cytometric analysis, and molecular/cytogenetic/FISH (fluorescent *in situ* hybridization) studies. The presence of immature (blastic) lymphoid cells in the peripheral blood indicates acute lymphoblastic leukemia/lymphoma (Chapter 8), which can be confirmed and further subtyped as B cell or T cell type by flow cytometry.

CHAPTER 10:

PLASMA CELL DISORDERS

Key Concepts:

- Normal plasma cell morphology, function and development
- Immunoglobulin structure and function
- Biology, pathophysiology, major clinical and laboratory features of:
 - Monoclonal gammopathy of uncertain significance
 - Multiple myeloma
 - Waldenstrom macroglobulinemia
 - Amyloidosis

Learning Objectives:

1. Describe the morphology; location, biology, and function of normal plasma cells in the human body.
2. Describe structure of immunoglobulins and the differences among the various immunoglobulin classes.
3. Describe the following laboratory tests used to analyze immunoglobulins in serum and urine and explain the clinical utility of each test.
 - total protein
 - protein electrophoresis
 - immunofixation
4. Describe the epidemiology, pathophysiology, morphology of blood and marrow, natural history, and major clinical and laboratory features of multiple myeloma (MM).
5. Describe the epidemiology, pathophysiology, morphology, natural history, and major clinical and laboratory characteristics of Waldenstrom macroglobulinemia (WM). Compare and contrast WM with MM.
6. Define amyloid and describe the pathophysiology, morphology, natural history, diagnostic criteria, and complications of light chain amyloidosis.
7. Define monoclonal gammopathy of undetermined significance (MGUS) and explain how MGUS differs from MM.

I. Plasma Cells

A. Function

Plasma cells are terminally differentiated B lymphocytes that perform the critical function of secreting antibodies in response to antigenic stimulation.

B. Morphology

Plasma cells (Fig 10.1) have a distinctive appearance, and are the most easily recognized of all bone marrow cells. The plasma cell is 2-3 times the size of a small lymphocyte with a round nucleus that is eccentrically placed (off to one side). The chromatin clumps tend to give the nucleus a “clock face” appearance. The Golgi apparatus creates a pale area adjacent to the nucleus surrounded by deep blue cytoplasm.

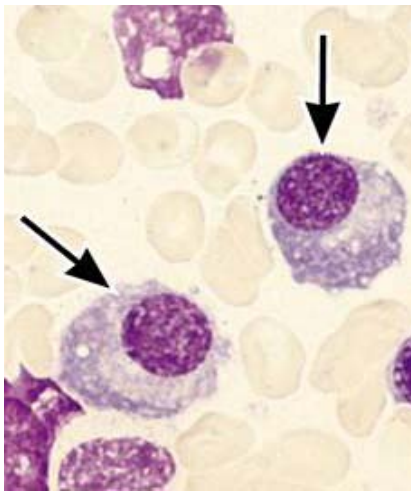


Figure 10.1. Plasma cells in a marrow aspirate smear. Note the abundant cytoplasm, eccentric round nucleus, clumped nuclear chromatin and paler Golgi structure adjacent to the nucleus.

C. Location

Plasma cells are normally found in lymph nodes, bone marrow, mucosa, spleen, and other tissues with lymphoid cells and are not normally found in the blood.

D. How B Cells become Plasma Cells

Plasma cells are terminally differentiated B cells. As discussed in the preceding chapter, the key feature of B cell development involves the rearrangement of the immunoglobulin heavy and light chain genes to create a unique antibody molecule that is expressed by a single B cell and all of its “offspring”. Expression of surface immunoglobulin is a characteristic of mature B cells. The first mature B cells are referred to as “naïve”. These small naïve mature B cells circulate in the peripheral blood and also make up the primary follicles and mantle zone of lymph nodes and other lymphoid tissue.

After stimulation by a specific antigen, some naïve B cells differentiate into rather short-lived IgM-producing plasma cells. Others proliferate as follicle center cells (FCC) within reactive germinal centers. Their interactions with dendritic cells and activated, antigen-specific T cells

result in stimulation of the B cells which then proliferate and undergo class switching to IgG, IgA, or IgE-producing cells. FCC also undergo somatic mutations of the immunoglobulin heavy and light chain variable regions (antigen binding site) that alter the affinity of the antibody for the antigen. Cells that emerge from this process producing antibody with low affinity for the specific antigen die, while cells producing antibody with the greatest antigen affinity survive. Some become memory cells and some become plasma cells. Most of these long-lived plasma cells migrate to the bone marrow where they produce their idiotype –specific antibody.

A plasma cell “clone” is a group of plasma cells all of which secrete an identical immunoglobulin molecule.

Different stages in the progression of B cell maturation are associated with the expression of specific antigens (see Chapter 9, Table 9.1). Plasma cells express cytoplasmic but not surface immunoglobulin, and lose many typical B cell markers such as CD20. Plasma cells are notable for their high expression of CD38 and CD138 (syndecan-1). These various cellular markers help pathologist identify a tumor’s cell of origin, which may not be evident by morphology alone. Knowing whether the malignancy is of lymphocyte vs plasma cell vs. lymphoplasmacytic cell origin is very important to the clinician when selecting the proper chemotherapy.

II. Immunoglobulins

A. Structure and Classes

Five types of heavy chains (γ = gamma, μ = mu, α = alpha, δ = delta, and ϵ = epsilon) determine the five Ig classes: IgG, IgM, IgA, IgD, and IgE (Table 10.1). There are two types of light chains, κ (kappa) and λ (lambda).

| Property | IgG | IgA | IgM | IgD | IgE |
|--|----------|----------|---------|----------|------------|
| Serum concentration mg/dl | 800-1400 | 100-300 | 60-200 | 2-4 | .02-.04 |
| Molecular weight | 150,000 | 160,000 | 900,000 | 180,000 | 190,000 |
| Carbohydrate (%) | 3 | 5-10 | 12 | 10-12 | 1 |
| Half-life (days) | 23 | 5.8 | 10 | 2.8 | 2.3 |
| Fixes complement? | + | 0 | +++ | 0 | 0 |
| Crosses placenta? | yes | no | no | no | no |
| Heavy chain | γ | α | μ | δ | ϵ |
| Light chain (κ : λ) ratio | 2:1 | 1:1 | 3:1 | 1:4 | ? |

Table 10.1. Comparative properties of human immunoglobulins. There are a number of subtypes of IgG with different propensities for complement and FcR binding, but this is beyond the scope of the course.

The basic immunoglobulin molecule (Fig 10.2) consists of two identical H (heavy) chains and two identical light chains. Two identical antigen binding sites result from the pairing of the variable regions of one heavy and one light chain. The Fc portion of the immunoglobulin molecule is formed from the two heavy chains and determines the properties of each class of antibody, including ability to bind complement and attach to effector cells (macrophages or neutrophils, which have Fc receptors). IgG, IgD, and IgE are monomeric forms of the basic Ig

molecule, IgM is normally a pentamer (5 sets of 2 μ chains and 2 light chains) and IgA forms monomers and dimers.

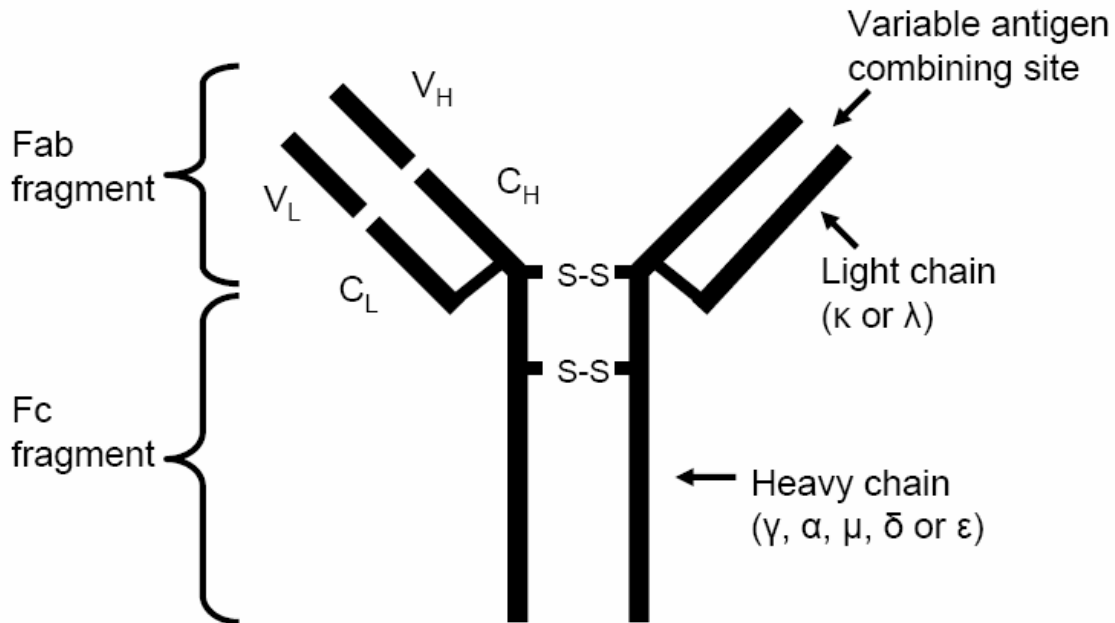


Figure 10.2. Schematic of Ig molecule. Variable portions determine antibody specificity. The type of heavy chain determines the class (IgG, IgA, IgM, IgD, or IgE). The Fc portion of the heavy chains binds receptors on macrophages.

B. Location

- IgG, the most abundant immunoglobulin of blood, is also present in tissue spaces and is the only immunoglobulin class that can cross the placenta.
- IgM is mainly confined to the blood and is produced in part by clones making early responses to antigen challenge.
- IgA is present in the blood, on epithelial surfaces and on IgA-producing B cells. Dimers of IgA with an additional secretory component are secreted into the intestine, bronchi, milk, saliva, or tears.
- IgD is present in the blood at low levels and is principally found on the surface of mature, naive B cells.
- IgE is present in the blood at low levels and is principally bound to mast cells.

C. Laboratory Tests to Assess Immunoglobins

Immunoglobulins are identified, characterized, and quantified by the following methods:

- Protein electrophoresis (serum and urine)
- Immunofixation (serum and urine)
- Quantitative assays (serum).

Total protein of serum or of urine is a quantitative test for all proteins found in the blood stream, including immunoglobulins. Therefore high levels of immunoglobulin usually results in high levels of total serum protein.

1. Protein electrophoresis

Electrophoresis is a useful screening test for detecting quantitative or qualitative abnormalities in serum or urine proteins. It is particularly useful in detecting monoclonal immunoglobulin. Serum or urine is applied to a surface (such as agarose gel) in a buffer. An electric current separates the proteins on the basis of mobility due to size and charge. The normal pattern in serum protein electrophoresis (SPEP) is presented in figure 10.3. The first peak (left) represents albumin, which has the highest mobility. The globulin peaks are designated alpha1, alpha2, beta, and gamma (the far right peak). The gamma peak contains mostly IgM and IgG and may contain IgA. IgA is also found in the beta globulin fraction. Note that the γ peak, while it does contain most of the circulating IgG (a.k.a. gamma globulin), is not named for this fact, but rather because it is the third major peak after alpha and beta.

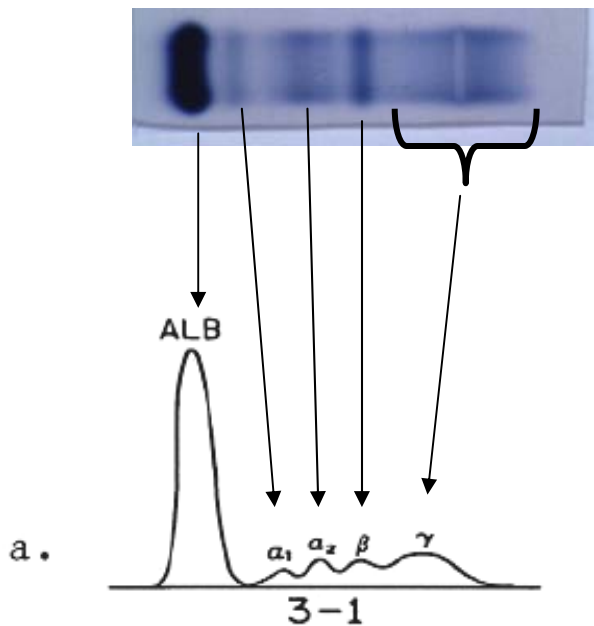


Figure 10.3. Serum protein electrophoresis (SPEP) showing normal pattern of protein bands in the gel (top) and protein peaks in the densitometer tracings (bottom). From left to right, albumin and α_1 , α_2 , β , and γ globulin bands and peaks are visible. Origin (where sample is applied) is at far right. Migration is toward the + pole (far left).

FYI

Other serum proteins that are found in the various globulin fractions include alpha-1 antitrypsin (alpha1), haptoglobin (alpha2), antithrombin III, transferrin, complement factor C3 (beta), plasminogen, and C-reactive protein (gamma). Fibrinogen, which is present in plasma but not normal serum, would migrate with the gamma globulins.

After the gel is stained to visualize the protein bands, a densitometric scanner generates tracings (Fig. 10.3) and the amount of protein in each peak can be estimated from the area under the peak, and reported as a percentage of total serum protein.

Normal serum contains polyclonal immunoglobulin, which by virtue of the wide variation in amino acid composition associated with different antibody isotypes generates a rather broad gamma globulin peak. **The presence of a narrow peak in the gamma region indicates the presence of an abnormally large amount of immunoglobulin that is homogeneous in terms of amino acid composition and structure; this in turn implies the presence of an abnormally large clone of plasma cells.** Such a monoclonal immunoglobulin component is variously referred to as an M protein, M component, M spike, or paraprotein. The pattern seen in patient with a monoclonal gammopathy is presented in Fig. 10.4. The narrow peak in the γ region (corresponding to the dense band in the electrophoretic gel) represents monoclonal immunoglobulin. It is important to note that protein electrophoresis does NOT tell us the immunoglobulin class of the monoclonal protein. This is determined by the immunofixation procedure.

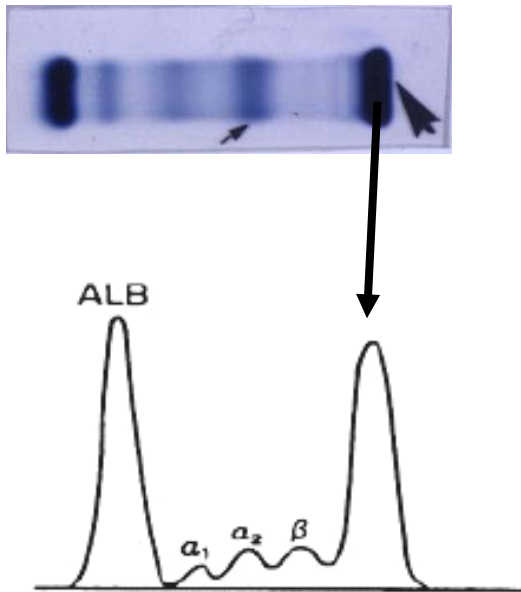


Figure 10.4. SPEP showing monoclonal gammopathy. The sharp narrow peak in the gamma region (long arrow) is the abnormal Immunoglobulin. This test cannot distinguish the Immunoglobulin class, which is determined by the immunofixation procedure.

Other abnormal SPEP patterns are shown in Figures 10.5 A (hypogammaglobulinemia) and 10.5 B (polyclonal gammopathy, characteristic of an inflammatory response).

Normal urine only contains a minimal amount of albumin. Monoclonal light chains (historically called Bence Jones protein) that are usually present in individuals with clonal plasma cell disorders are excreted in the urine, and are detected by urine protein electrophoresis (UPEP) (Fig 10.5 C).

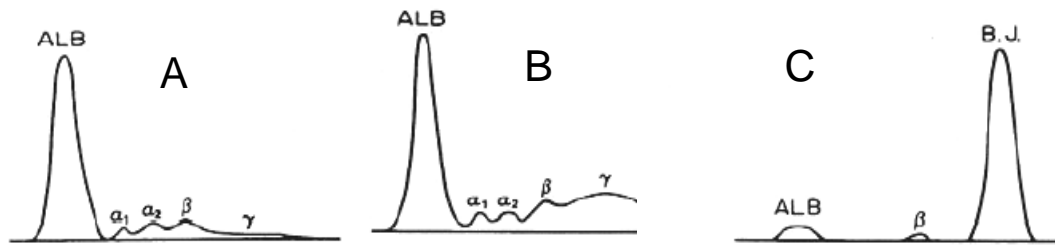


Figure 10.5. A. SPEP showing hypogammaglobulinemia. B. SPEP showing polyclonal hypergammaglobulinemia. C. UPEP with monoclonal light chain (Bence Jones protein)

2. Immunofixation

Immunofixation is a technique that allows identification of the immunoglobulin class (IgG, IgM, IgA) and the type of light chain (kappa or lambda) in a monoclonal immunoglobulin. The proteins in the sample (serum or urine) are separated using standard electrophoresis. The electrophoretic gel is then exposed to an antibody (anti-IgG, anti-IgA, anti-IgM, anti-kappa, or anti-lambda) that binds to and immobilizes (fixes) its target immunoglobulin class or light chain in the gel. The unfixed protein is washed out and the gel is stained for protein. Figure 10.6 A shows the result of an immunofixation assay done on normal serum. Figure 10.6 B shows the result of a similar assay done on serum containing a monoclonal immunoglobulin.

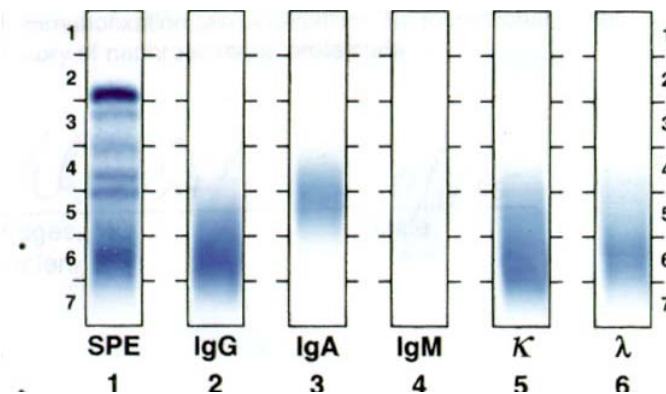
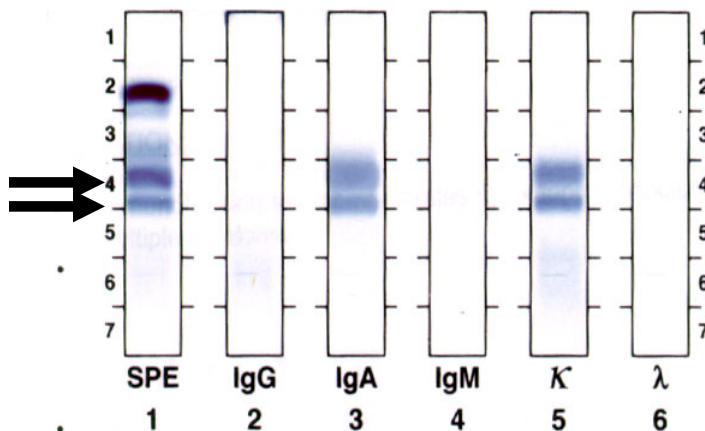


Figure 10.6 Immunofixation

A. Immunofixation of normal serum. Lane 1 is the SPEP gel. Gels in lanes 2-6 have been fixed with antisera to the heavy and light chains indicated.



B. Immunofixation showing monoclonal IgA kappa in serum. Note the two prominent narrow bands in the beta region in the SPEP (lane 1, arrows) and the decreased level of normal Ig in the gamma region. Both abnormal bands fix with antisera to IgA (lane 3) and kappa (lane 5). They represent IgA in monomer and dimer forms.

3. Quantification of M-proteins

- **The area under the curve on the SPEP and UPEP are used to quantitate the monoclonal protein. Following the amount of abnormal immunoglobulin is the best way to follow the progression of the myeloma and its response to treatment, since the amount of M-protein is usually a good reflection of tumor burden.**

III. Plasma Cell Disorders

A. Multiple Myeloma (MM) -- Also Called Plasma Cell Myeloma:

Archeologists long ago unearthed prehistoric skeletons with the characteristic bone lesions of multiple myeloma. However, little was known about the disease until the middle of the 19th century when Dr. Henry Bence Jones examined the urine from a patient with destructive bone disease and found a protein with the unusual property of precipitating at 56 °C and dissolving at the boiling point. This protein was later shown to be the isolated light chain of the immunoglobulin molecule. In recent years a great deal of progress has been made in the treatment of this malignancy.

1. Definition

MM is a malignancy of plasma cells. The clinical problems associated with MM result from:

- Bone marrow infiltration by the malignant plasma cells
- Production of large quantities of functionless monoclonal immunoglobulin and/or monoclonal free light chain.
- Decreased production of normal immunoglobulins
- Bone destruction by cytokines produced by the malignant plasma cells

2. Epidemiology

MM predominantly affects older patients and rarely occurs in people younger than 40 years old. The incidence is 3-10 per 100,000 people per year and the incidence rises sharply with age. MM is more common in men and in African Americans. The incidence has been increasing over the past several decades. MM is the second most common hematologic malignancy in the US (following non-Hodgkin lymphoma). It represents about 1% of all cancers, and 13% of hematologic cancers.

3. Etiology

As is the case with most forms of cancer, a single cause cannot be identified in most cases. Several risk factors have been identified, including radiation exposure, exposure to chemicals such as pesticides or herbicides, and genetics (the risk of MM is higher in people who have close relatives with the disease). Patients with monoclonal gammopathy of uncertain significance (MGUS – see discussion below) develop myeloma at the rate of about 1% per year. MM development is postulated to follow the two hit hypothesis:

- Antigenic stimulation causes expansion of multiple benign clones;
- Mutagenic events in a dividing cell cause malignant transformation. The primary mutagenic event is thought to be a translocation involving an immunoglobulin gene and an oncogene, similar to mutations found in some B-cell lymphomas (see Chapter 9).

4. Clinical presentation

The first signs and symptoms may not occur until the disease has reached a fairly advanced stage. They often include fatigue, back pain, recurrent infections, kidney damage and hypercalcemia..

5. Pathophysiology (Figure 10.7)

- **Malignant plasma cells replace normal bone marrow**, causing anemia and often neutropenia and/or thrombocytopenia with all of their attendant complications.
- **Extramedullary extension of plasma cells can form tumors** in soft tissue (plasmacytomas). Spinal cord compression from plasma cell masses protruding posteriorly from the vertebral bodies requires emergency radiation to prevent the devastating complication of paraplegia..
- **Abnormal immunoglobulin accumulates in serum, and free light chains are filtered into the urine.** About 80% of patients with myeloma secrete a whole immunoglobulin molecule (either IgG or IgA) in excessive amount. This may be accompanied by extra light chain which is either kappa or lambda light chain restricted. **About 20% of patients secrete only kappa or lambda light chains and are thus said to have light chain myeloma.** Because light chains are filtered into the urine, the serum protein electrophoresis (SPEP) will not have a large monoclonal spike. It will show a decreased amount of gamma globulin because of the suppression of normal immunoglobulin production that occurs in myeloma. Urine protein electrophoresis (UPEP) will show the monoclonal spike.
- **The monoclonal protein may cause a variety of complications, including:**
 - 1) Precipitation of light chains in renal tubules causing renal damage.
 - 2) Paraprotein may coat platelets or interfere with clotting factor function, causing bleeding.
 - 3) Amyloid formation (See section on amyloidosis below)
 - 4) Hyperviscosity of blood at very high protein levels can occur (this is more common in Waldenstrom macroglobulinemia – see below).
 - 5) Peripheral neuropathy
- **Myeloma cells produce cytokines that stimulate osteoclast growth and inhibit osteoblast differentiation, leading to destruction of bone. Complications include:**
 - 1) Bone pain
 - 2) Osteoporosis/osteopenia
 - 3) Lytic bone lesions
 - 4) Pathologic fractures
 - 5) Hypercalcemia
 - 6) Renal insufficiency
 - 7) Mental status change.
- **Decrease in normal plasma cell function and antibody production result in immunodeficiency and increased risk of bacterial infection.**

As you can see from the outline above, the clinical manifestations of myeloma are protean. About 70% of patients initially present with back pain. Because of the many possible affronts to the kidneys, including precipitation of light chains in renal tubules, hypercalcemia, amyloid deposition, infection and hyperviscosity, renal failure is also common at presentation. Thus, in an adult who presents with back pain, the presence of cytopenias, renal failure or hypercalcemia should alert you to the possibility of multiple myeloma.

PATHOPHYSIOLOGY OF MULTIPLE MYELOMA

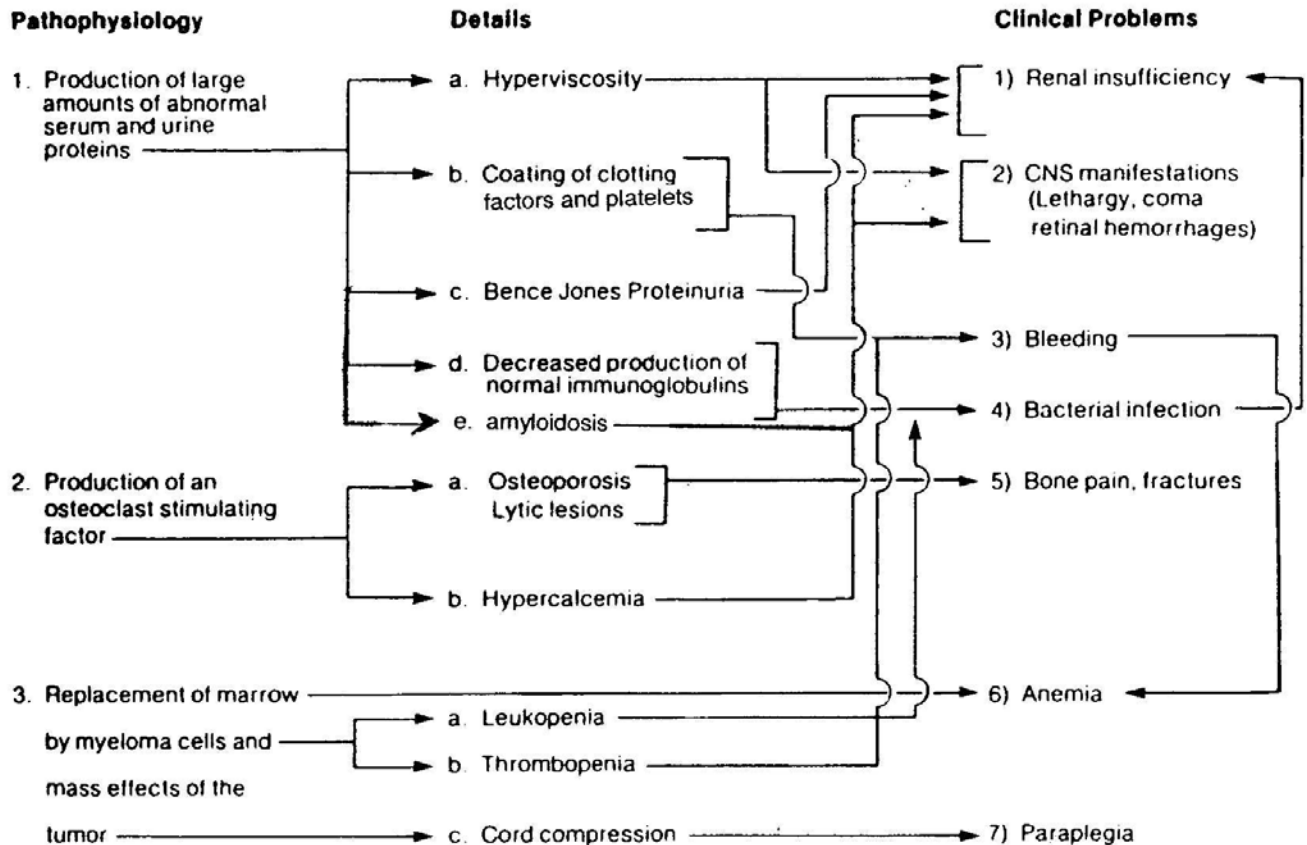


FIGURE 10.7 Pathophysiology of multiple myeloma

6. Laboratory findings

- CBC: Anemia, often pancytopenia
- Blood smear: rouleaux formation (stacking of red cells coated by immunoglobulin). (Fig 10.8)
- Total serum protein: elevated because of the monoclonal protein
- Serum calcium: hypercalcemia
- Erythrocyte sedimentation rate (ESR): very high due to coating of RBC by the monoclonal protein

- Blood viscosity: high when monoclonal protein levels are very high
- SPEP: Low levels of normal immunoglobulin and presence of a monoclonal protein (Fig 10.4)
- Urine electrophoresis: monoclonal light chains (Fig 10.5)

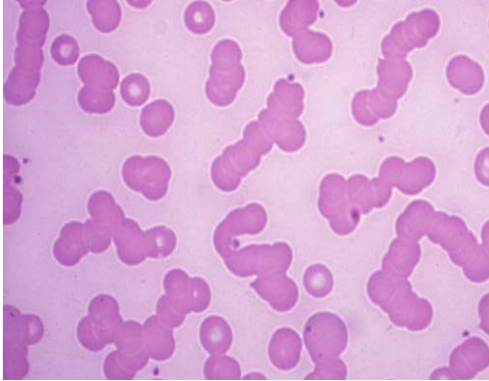


Figure 10.8. Blood smear showing rouleaux formation. Red cells, which are disc shaped, are stacked together. (“rouleaux” comes from French “roll”, as in “roll of coins”). This happens when the RBC are coated with protein, as occurs in plasma cell disorders when there is a high monoclonal protein level. The normal surface charge of the red blood cell is neutralized by the excess protein; the cells do not repel each other. In contrast to rouleaux, “agglutinated” red blood cells form clusters (not stacks) that are brought together by IgM reacting with antigens on red cell surface.

Additional laboratory studies are performed when MM is suspected.

Immunofixation of serum is used to identify the serum monoclonal protein. Results of SPEP, UPEP, and immunofixation in patients with myeloma are as follows:

- Almost 60% have monoclonal IgG.
- 20% have monoclonal IgA.
- There is free monoclonal light chain in the serum and urine in most patients, since myeloma cells tend to secrete excess light chains along with intact immunoglobulin.
- Almost 20% have **only** monoclonal light chain (this is called light chain myeloma). Light chains are filtered by the glomerulus, so most of this will appear in the urine. The UPEP is diagnostic. Typically the SPEP in light chain myeloma shows just hypogammaglobulinemia and no monoclonal spike (Fig 10.5 A).
- IgM production is characteristic of Waldenstrom macroglobulinemia (WM – see below) and is very rare in MM.
- Rarely there is no detectable M protein in serum or urine. This is called “non-secretory” myeloma; the malignant cells either make Ig but are unable to secrete it, or have a severely disrupted Ig genetic locus that produces no protein.

7. Bone marrow aspiration and biopsy

- Increased numbers of plasma cells (normal number is < 4%), often with abnormal morphology
- Immunohistochemistry or flow cytometry demonstrate that the plasma cells express only kappa or only lambda light chains, indicating a clonal population of cells.
- Cytogenetic analysis often shows characteristic translocations, many of which involve the H chain locus on 14q23. As is the case in other hematologic malignancies, these have prognostic significance.
- Some cases exhibit amyloid deposition around vessels (see below). This is demonstrated by Congo red staining.

8. Radiologic findings (Fig. 10.9)

- osteoporosis
- osteolytic lesions
- pathologic fractures
- compression of vertebral bodies

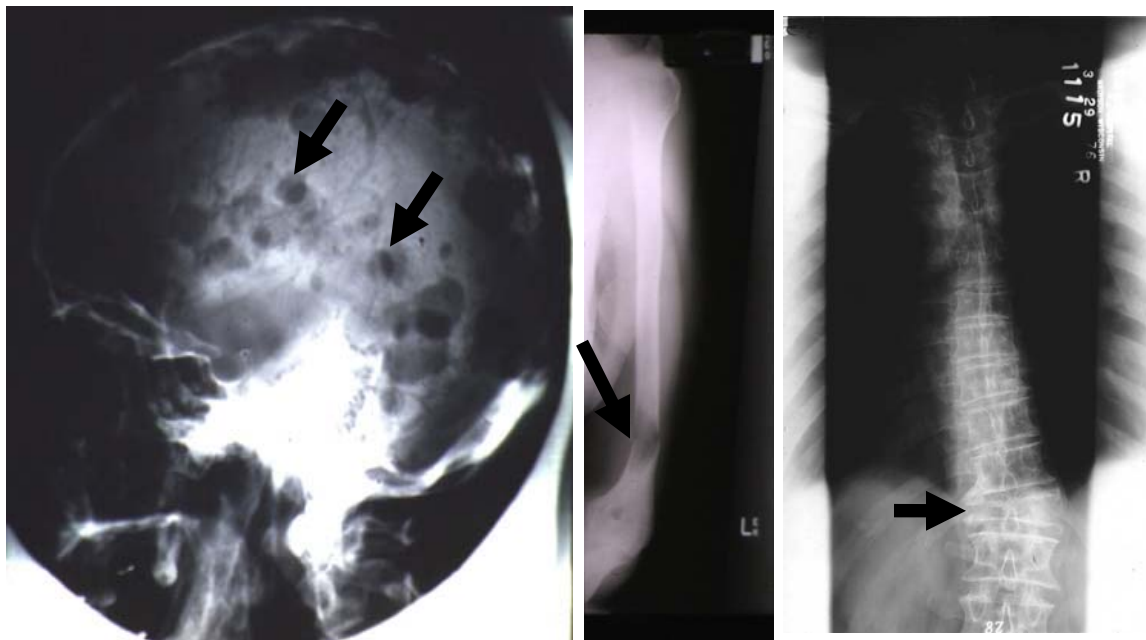


Figure 10.9. Bone lesions in multiple myeloma. Left panel, osteolytic lesions in the skull. Center panel, lytic lesion in humerus causing pathologic fracture. Right panel, vertebral compression fracture.

9. Diagnostic criteria for multiple myeloma:

- M protein in serum or urine
- Bone marrow containing > 10% clonal plasma cells, or presence of extramedullary (i.e., outside the marrow) plasmacytoma
- Related organ or tissue impairment (CRAB: hyperCalcemia, Renal insufficiency, Anemia, Bone lesions)
- Some patients have monoclonal gammopathy with increased numbers of monoclonal plasma cells in the marrow, but no symptoms or evidence of tissue injury. They are considered to have “**smoldering myeloma**” and are usually followed closely but not treated unless the disease progresses.

10. Treatment

- Chemotherapy, radiotherapy to destroy malignant cells
- Bisphosphonates (pamidronate, zoledronate) to slow bone destruction and treat hypercalcemia
- Pain control and other palliative measures

- Protecting the kidneys by avoiding dehydration and potentially nephrotoxic drugs
- Autologous stem cell transplant

11. Prognosis

- MM is generally incurable and the median survival is about 5 years.
- Newer combination therapies that include thalidomide or its analogs (immunomodulating agents), steroids, bortezomib (a proteasome inhibitor) in conjunction with alkylating agents and autologous marrow transplantation have significantly improved the median survival in myeloma.

12. Common causes of death include

- Bone marrow failure and related complications (e.g., infection)
- Renal failure
- Hypercalcemia

B. Plasmacytoma

Plasmacytomas are tumors consisting of clonal plasma cells which may occur in the bone (osseous) or outside of the bone (extraosseous). Extraosseous lesions may occur as an extension of intramedullary myeloma or as a solitary lesion unassociated with the marrow space. The majority of solitary lesions occur in the upper respiratory tract. The diagnosis of plasmacytoma is made by biopsy of the tumor mass and examination for clonal plasma cells. After the diagnosis is made, laboratory and radiologic tests are necessary to exclude concurrent MM. Plasmacytomas are treated with radiation. Osseous plasmacytomas often progress to MM. Development of MM in patients with extraosseous plasmacytoma after radiation treatment is uncommon.

C. Waldenstrom's Macroglobulinemia (WM)

Waldenstrom's macroglobulinemia is a relatively uncommon low grade malignancy of lymphoplasmacytic cells (B lymphoid cells with some plasma cell features). It affects older individuals, with a mean age at diagnosis in the upper 60s. It is characterized by secretion of a very large amount of nonfunctional monoclonal immunoglobulin, which in this case is IgM.

1. Waldenstrom's macroglobulinemia contrasted with Multiple Myeloma:

- **Waldenstrom's macroglobulinemia is a malignancy of lymphoplasmacytic cells that secrete monoclonal IgM** whereas MM is a malignancy of plasma cells that secrete monoclonal IgG, IgA, or Light chains.
- **WM is a low-grade non-Hodgkin's lymphoma**; MM is a more aggressive malignancy of plasma cells. Both are usually incurable, but WM tends to have a more indolent course.
- **WM (unlike typical myeloma) may infiltrate lymph nodes and spleen causing lymphadenopathy and splenomegaly.**
- **Hyperviscosity syndrome occurs in WM when there is a high blood level of IgM** (usually over 6 g/dL). IgM, because of the very large size of the molecule, makes a disproportionate contribution to blood viscosity at any given concentration. Hyperviscosity is less common in Myeloma but can occur occasionally, especially in

IgA Myeloma as IgA tends to polymerize where as IgG does not. Hyperviscosity causes sludging within the vasculature that may result in:

- CNS manifestations (lethargy, coma, headaches)
- Blurred vision, retinal hemorrhages
- Stroke
- Increased plasma volume and congestive heart failure
- WM (unlike Myeloma) does **not** cause lytic bone disease, which is a major cause of clinical symptoms in Myeloma.
- WM (unlike Myeloma) does not often cause renal disease because the malignant cells do not secrete much free light chain.
- WM (unlike Myeloma) is sometimes associated with:
 - **Cold agglutinins**: a situation in which the monoclonal IgM is an antibody to a red cell surface antigen. This may result in hemolysis and/or Raynaud's phenomenon (cold-induced cyanosis of digits due to antibody-mediated sludging of blood). In severe cases, gangrene of fingers and toes may occur.
 - **Cryoglobulinemia**: A form of vasculitis caused by circulating cold-insoluble immune complexes containing monoclonal IgM.
- Both WM and MM infiltrate the marrow causing **significant anemia** and sometimes pancytopenia. In Waldenstrom's some of the anemia is apparent rather than real, as the large size of the IgM molecule exerts an osmotic effect which increases plasma volume. The increase in plasma volume may cause heart failure.
- Both WM and MM may cause non-specific coating of platelets and clotting factors by monoclonal protein resulting in **bleeding**.

2. Laboratory abnormalities in WM

- CBC: Anemia, pancytopenia
- Blood smear: rouleaux formation (Fig. 10.8) or (if the IgM is acting as a cold agglutinin) agglutination (clumping) of RBC
- Serum protein: high
- SPEP: Monoclonal spike, usually in gamma region
- Immunofixation: Monoclonal IgM
- Bone marrow or lymph node biopsy: lymphoplasmacytic B cells expressing monoclonal IgM

3. Criteria for diagnosis

- Biopsy (marrow or lymph node) showing monoclonal (i.e., expressing only κ or λ light chain) lymphoplasmacytic B cells
- Monoclonal IgM in serum

4. Treatment

- WM is a low grade lymphoma and is treated only when the patient is symptomatic. Various forms of **chemotherapy** are used, including anti-CD20 antibody (rituximab).
- **Plasmapheresis** (plasma exchange) effectively removes intravascular IgM until more definite chemotherapy becomes effective. It may be life saving for patients with severe CNS symptoms (coma) due to hyperviscosity. Plasmapheresis is much less effective in Myeloma because IgG and IgA, unlike IgM, are also in extravascular

compartments and quickly re-equilibrate with the intravascular compartment after plasmapheresis

5. Prognosis

WM is an indolent lymphoma and is not curable. Median survival is 5-10 years.

D. Amyloidosis

1. Description

Amyloidosis is a heterogeneous group of disorders resulting from the deposition of a very stable, insoluble protein in a characteristic fibrillar **β -pleated sheet structure**. It may be found in a wide array of body tissues. Any of at least 25 different proteins may assume this configuration. The result is organ enlargement and organ damage. Amyloid binds a dye called Congo red and exhibits green birefringence (splitting into two unequally reflected waves) when viewed under polarized light, which aids in the diagnosis.

2. Forms of amyloidosis.

The two common forms of amyloid are:

- 1) **Light chain amyloidosis** occurs when a clone of plasma cells (or rarely lymphoplasmacytic cells) secretes a clonal immunoglobulin **light chain** that happens to have the physicochemical ability to form beta pleated sheets. The light chain is usually **lambda** light chain, which is processed by macrophages to form amyloid. Amyloid derived from light chain is called **AL amyloid**
- 2) **Secondary amyloidosis** does not result from plasma cell secretion of light chain.. This form of amyloid (AA) is derived from a circulating protein called Amyloid A protein, which acts an acute phase reactant. AA amyloid is associated with chronic inflammatory conditions such as juvenile rheumatoid arthritis or Familial Mediterranean Fever (FMF).

FYI

Other forms of amyloidosis, and their associated amyloidogenic proteins, include:

- Hemodialysis (beta-2 microglobulin)
- Alzheimer's disease (beta-amyloid protein)
- Inherited disorders (mutations in transthyretin and other plasma proteins)
- Endocrine disorders (e.g., medullary thyroid cancer – calcitonin)
- Prion disease (prion protein)

3. Light chain (AL) amyloidosis

- **Incidence**

- Most common form of amyloidosis (but still uncommon)
- Median age at diagnosis is 64 years
- More common in men

- Approximately 20% of AL amyloidosis patients have Multiple Myeloma. 80% do not have overt myeloma but do have a demonstrable serum or urine monoclonal light chain.
 - About 10% of Multiple Myeloma patients develop amyloidosis
- **Clinical findings** result from organ involved and include:
 - **Heart:** cardiomegaly and congestive heart failure
 - **Liver:** hepatomegaly
 - **Kidney:** nephrotic syndrome, renal failure
 - **Tongue:** macroglossia (large tongue)
 - **Skin:** purpura, particularly facial and periorbital, resulting in “raccoon eyes”
 - **Spleen:** splenomegaly
 - **Ligaments:** carpal tunnel syndrome
 - **Nerves:** peripheral neuropathy
 - **GI tract:** malabsorption, diarrhea, bleeding
- **Diagnosis**
There is no blood test, radiograph or scan diagnostic for amyloidosis. Diagnosis requires tissue biopsy (gingival, rectal, bone marrow, kidney, subcutaneous fat) stained with Congo red. Amyloid is often seen within vascular walls. In light chain amyloidosis, light chain is found in the serum or urine.
- **Treatment**
Treatment of AL amyloidosis is similar to treatment of Myeloma, but is often less successful. Treatment of secondary (AA) amyloidosis is directed toward control of the underlying inflammatory disease. In the Middle East, amyloidosis associated with FMF responds to colchicine.
- **Prognosis**
Prognosis is quite variable, but many patients die of the disease within 1-2 years of diagnosis. Cardiac amyloidosis has a particularly poor prognosis.

E. Monoclonal Gammopathy of Undetermined Significance (MGUS)

1. Description and Incidence

SPEPs and UPEPs are often ordered when patients are being evaluated for renal, neurologic, or rheumatologic diseases as well as for low blood counts. Using the very sensitive laboratory methods now available, monoclonal immunoglobulin (M protein) can be detected in the blood or urine of at least 5% of all persons over the age of 70. Most of these individuals do not have a malignant disorder, but instead have a condition called monoclonal gammopathy of undetermined significance (MGUS). The M protein can be IgG, IgA, or IgM.

2. Diagnosis

MGUS is diagnosed when 1) a patient has monoclonal gammopathy and 2) MM, lymphoma, and amyloidosis have been ruled out. Diagnostic criteria include:

- M protein <3g/dL
- bone marrow plasma cell level <10%
- no lytic bone lesions
- no MM related organ or tissue impairment (hypercalcemia, renal insufficiency, anemia, bone lesions)
- no evidence of B cell lymphoma or amyloidosis

3. Clinical course

The clinical course in most persons with MGUS is not progressive, but some patients do go on to develop overt Multiple Myeloma, Waldenstrom's macroglobulinemia or amyloidosis. The risk of progression is about 1% per year. The median interval from MGUS recognition to overt disease is 10 years. Individuals with MGUS do not require treatment.

CHAPTER 11

Part 1: OVERVIEW OF HUMAN HEMOSTASIS

Key Concepts:

- Functions and essential components of the coagulation and fibrinolytic systems
- Mechanisms by which coagulation and fibrinolysis are regulated

Learning Objectives:

1. Describe the role of platelets in the hemostatic process and the relationship between platelet structure and function.
2. Describe the processes of platelet adhesion, activation, aggregation, and secretion. Explain why platelets do not adhere to blood vessels under normal circumstances.
3. Describe the interaction between platelets and elements of the coagulation cascade.
4. Describe the production and functions of von Willebrand factor (vWF), the role of desmopressin in regulating its plasma concentration, and the relationship between VWF multimer size and VWF function.
5. Describe the process of thrombin and fibrin formation, including the roles of tissue factor, factors VII, X, V, IX, VIII, prothrombin, fibrinogen, and factors XI and XIII.
6. Describe the role of vitamin K in coagulation.
7. Describe the regulation of the clotting system, including the roles of antithrombin/heparin, tissue factor pathway inhibitor, protein C and protein S, and the role of the endothelial cell.
8. List the enzymes involved in fibrinolysis; describe how fibrinolysis is regulated, and how the activation of plasminogen is localized to the fibrin clot.
9. (Chapters 11 and 12) List some laboratory tests used to evaluate the hemostatic and fibrinolytic systems and describe how each test is used clinically to evaluate bleeding disorders.
10. (Chapters 11 and 12) Be able to generate a differential diagnosis for abnormal results of the prothrombin time/INR and the activated partial thromboplastin time (aPTT).

I. The Human Hemostatic Response to Injury

A. Characteristics

1. A rapid and vigorous response to “plug the hole” and maintain intravascular volume

The human circulatory system, particularly on the arterial side, maintains relatively high pressures. When vascular integrity is breached due to injury, rapid vasoconstriction and formation of the platelet plug (**primary hemostasis**) minimize blood loss.

2. An explosive, localized increase in thrombin generation to trigger fibrin clot formation

Circulating coagulation factors generate thrombin via a proteolytic cascade. **Thrombin is central to the hemostatic response. It is a potent platelet activator and it converts**

soluble fibrinogen to insoluble fibrin clot. The activated platelet surface is an important site for assembly and localization of the membrane-bound enzyme complexes of coagulation. The resulting fibrin clot anchors the platelet plug (**secondary hemostasis**).

3. A highly regulated response to prevent uncontrolled thrombosis

In the absence of injury, blood flow must be maintained to ensure proper delivery of oxygen and nutrients. Normal endothelium prevents exposure of the blood to extravascular tissue factor and provides an antithrombotic surface to maintain flow in uninvolved vessels. Circulating coagulation inhibitors localize the hemostatic response to the site of injury and help to prevent spontaneous thrombotic events (myocardial infarction, stroke, venous thromboembolism). **Disruption of the endothelial barrier, loss of inhibitor function, and improper localization of the hemostatic response all may contribute to pathologic thrombosis.**

4. A response that transitions to clot remodeling (fibrinolysis) and wound repair

The original thrombus incorporates plasminogen, which when converted to plasmin promotes lysis of the fibrin clot. Fibrinolysis is confined to the clot by circulating inhibitors. The fibrin clot provides a matrix for inflammatory cell and fibroblast migration that is fundamental to wound repair.

B. Components

Effective hemostasis requires a balance between opposing prothrombotic and antithrombotic components of the injury response. The major components are defined below:

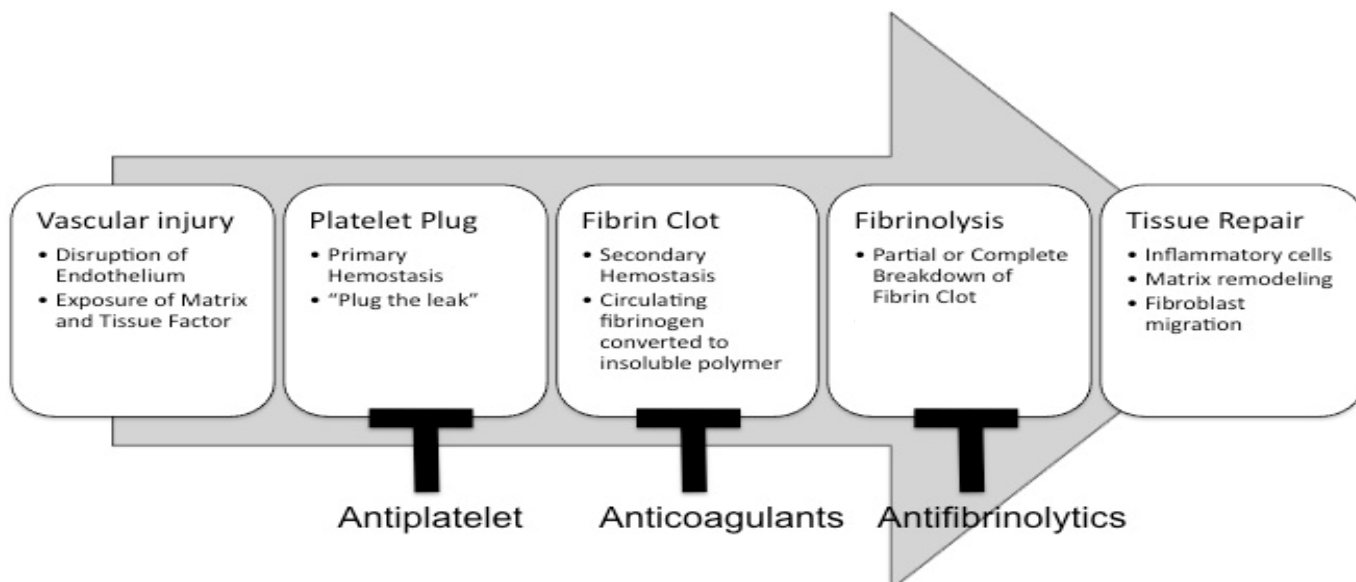
1. Prothrombotic components

- **Platelets/von Willebrand factor-** responsible for formation of the primary hemostatic plug.
- **Coagulation cascade-** a sequence of enzymatic reactions leading to thrombin generation and fibrin clot formation.
- **Fibrinolysis inhibitors-** soluble protease inhibitors that down-regulate fibrinolysis and prevent degradation of fibrin clot.

2. Antithrombotic components

- **Endothelium-** cells lining the blood vessels that possess anticoagulant, anti-platelet, and profibrinolytic properties, and act as a barrier to prevent exposure of the blood to tissue factor.
- **Fibrinolysis-** a second enzyme cascade that lyses and remodels the fibrin clot, and promotes wound healing.
- **Coagulation inhibitors-** soluble protease inhibitors that down-regulate the coagulation cascade and remove activated coagulation factors from the circulation.

C. Overview:



Overview of the hemostatic process. Classes of drugs that inhibit various steps in the process are shown below.

II. Primary Hemostasis: The Platelet-Vasculature Response

Platelets are responsible for primary hemostasis, the initial formation of platelet aggregates that plug the hole at the site of vascular injury. The physiology of platelet plug formation can be broken down into components of adhesion, activation, aggregation, and secretion.

- **Adhesion** is the initial interaction of platelets with non-endothelial surfaces, primarily governed by the binding of the platelet glycoprotein Ib-V-IX complex (**Gp Ib**) to von Willebrand factor (**vWF**) bound at the site of injury.
- Platelets are **activated** by a variety of agonists (thrombin, collagen, adenosine diphosphate, etc.) via specific membrane receptors, resulting in aggregation and secretion.
- **Aggregation** refers to the cross-linking of activated platelets, primarily mediated by fibrinogen binding to an activated conformation of the glycoprotein IIb/IIIa (**Gp IIb/IIIa**) complex.
- **Secretion** of platelet granules releases substances that recruit/activate additional platelets and locally amplify coagulation.
- Finally, **retraction** of the newly formed thrombus is a contractile event triggered by assembly of the multi-protein cytoskeleton of the platelet.

A. Platelets

1. Morphologic appearance

Platelets are small (2-3 μm mean diameter), anucleate cell fragments that are derived from bone marrow megakaryocytes. Platelets are normally present at approximately 150-450,000/ μl of blood (compared to 4-5,000,000 RBC/ μl and 4-10,000 WBC/ μl), and appear as granular bluish cell fragments on Wright stained blood smears. Platelets are adhesive, contractile entities that are activated by interaction with the subendothelial matrix and/or soluble agonists. Platelet activation results in shape change, conformational activation of the Gp IIb/IIIa complex, granule release, and eventually thrombus contraction.

FYI

Electron microscopy of the resting platelet (Figure 11.1) reveals important features including:

- the microtubular band (MT), which depolymerizes during shape change (pseudopod formation and contraction);
- α -granules, which contain chemokines (platelet factor 4), adhesive proteins (fibrinogen, von Willebrand factor, fibronectin), procoagulant proteins (factor V, factor XIII), and growth factors (platelet-derived growth factor);
- dense granules, which contain ADP, ATP, serotonin, calcium, and pyrophosphate;
- the surface connected (open) canalicular system, which provides a route for entry to platelet, granule release, and an internal store of membrane;
- the dense tubular system (DTS), which is a closed channel network of endoplasmic reticulum that sequesters calcium and synthesizes prostaglandins and thromboxanes.

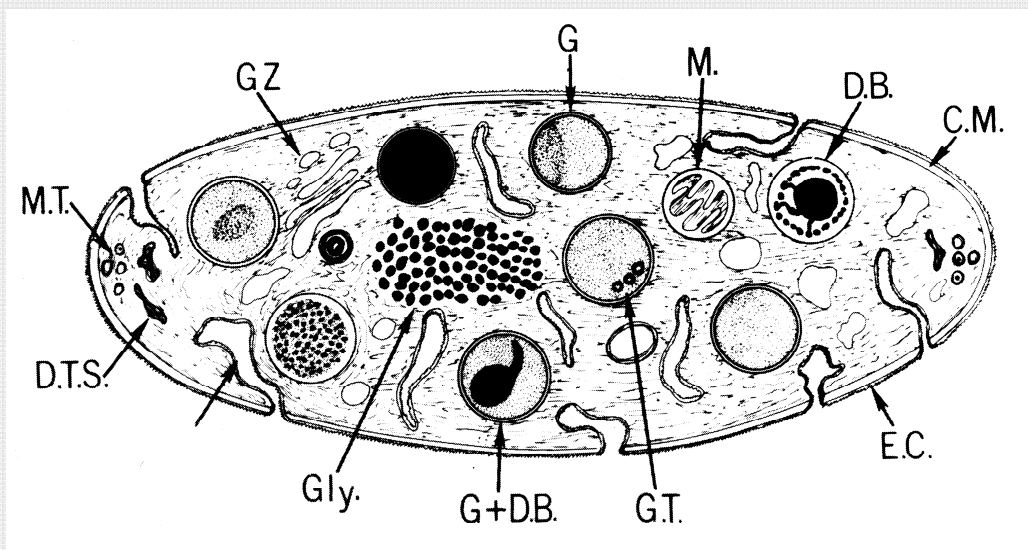


Figure 11.1- Discoid platelet cut in cross section. Components of the peripheral zone include the external coat (EC), and trilaminar unit membrane (CM). The matrix of the platelet contains the circumferential microtubular band (MT) and glycogen granules (Gly). The membrane systems include the canalicular system (CS), which connects to the surface (unlabeled arrow), and the dense tubular system (DTS). Formed elements include mitochondria (M), granules (G), Golgi zone (GZ) and dense bodies (DB). (From Colman RW et al.: *Hemostasis and Thrombosis*, Philadelphia, JB Lippincott Co., 2nd Edit., pg. 344).

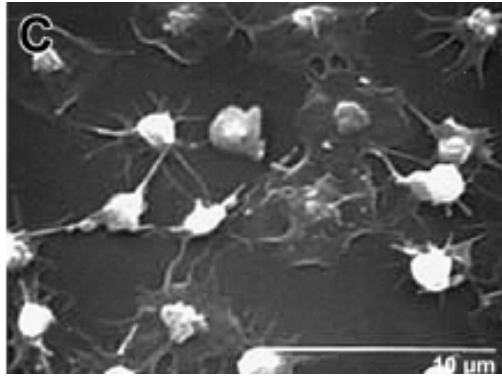


Figure 11.2- Platelet Shape Change. Adherent platelets demonstrate filopodia (finger-like projections) formation and spreading across a collagen-coated surface (From: Patel, D et al, *Blood* 2003;101:929-36).

2. Platelet life cycle

Platelets are generated from megakaryocytes, which are very large (20-25 μm diameter), polyploid (4N-64N), low abundance (0.1% nucleated cells) bone marrow cells. When mitosis ends during megakaryocyte differentiation, it is followed by endomitosis, an unusual process in which DNA replication occurs but the nucleus and cell do not undergo division. Normally, megakaryocytes will reach a state of 8N, 16N, or 32N ploidy before their cytoplasm is mature. Demarcation membrane channels then develop and divide the megakaryocyte cytoplasm into 1,000-3,000 platelets. **The production of platelets is largely mediated by the cytokine thrombopoietin (TPO).** TPO binding results in increased megakaryocyte number, size, and ploidy. **Released platelets survive for ~7-10 days in the circulation**, after which platelets that have not been consumed during clotting are removed by the spleen. About 30% of circulating platelets are normally sequestered in the spleen and can be released in response to epinephrine. The body strives to maintain a constant mass of platelets, such that there is generally an inverse relationship between platelet number and mean platelet volume (MPV). The spleen stores platelets in proportion to its size, which results in low circulating platelet counts without megakaryocyte hyperplasia in patients with splenomegaly. In contrast, when the platelet count is reduced due to rapid destruction/consumption by a normal sized spleen, megakaryocytic hyperplasia in the bone marrow and increased MPV of circulating platelets is expected. Thus, large circulating platelets on the blood smear in a patient with thrombocytopenia suggest peripheral destruction/consumption of platelets.

B. Structure and Life cycle of von Willebrand Factor (vWF)

1. Functions:

Von Willebrand Factor (vWF) is an adhesive protein that serves two critical hemostatic functions.

- **It is the major carrier protein for coagulation factor VIII** and significantly prolongs the plasma half-life of this factor. Thus, a reduced plasma level of vWF usually results in a parallel reduction in plasma factor VIII.

- **vWF is required for platelet tethering and adhesion to exposed subendothelial matrix under high shear (flow) conditions.** vWF is critical to formation of the platelet plug at the site of injury, especially in the arterial circulation.

2. Biosynthesis/Structure

vWF is synthesized by, and stored in, endothelial cells and megakaryocytes. It is also found extracellularly in subendothelial connective tissue. **vWF is assembled into large multimeric structures that are critical for platelet adhesion.** These vWF multimers can be as large as 20,000 kDa in size. vWF is released by endothelial cells into the blood. Its release is enhanced by secretagogues such as desmopressin acetate (DDAVP), an agent used in the treatment of von Willebrand disease (vWD). vWF in the circulation is normally folded in a way that prevents its binding to platelets. When bound to a surface that is exposed to flowing blood it unfolds, exposing its Gp Ib binding sites.

3. Extracellular proteolytic processing by ADAMTS13 (Figure 11.3):

vWF is secreted from endothelium or platelets in a range of multimer sizes, including ultra-large vWF multimers (> 20,000 kDa). These ultra-large molecules are very “sticky”: they have a tendency to unfold spontaneously and attach to platelets when exposed to high shear stress, or after interaction with the endothelial surface. Partially unfolded ultra-large vWF multimers that have bound to platelets are cleaved by the protease ADAMTS13 into smaller, less sticky multimers. This system can fail if there is inherited or acquired deficiency of ADAMTS13, leading to formation of large VWF-platelet aggregates that deposit in the microvasculature and cause tissue injury. This causes the disease thrombotic thrombocytopenic purpura (TTP) (discussed in Chapter 12).

Multimer size and structure are critical to the platelet adhesive properties of vWF under the high shear conditions typically found in the arterial circulation. Cleavage by ADAMTS13 down regulates vWF activity. Variations in this protease activity may influence the risk of thrombosis in the general population, although this has yet to be proven. Conversely, mutations in the von Willebrand factor gene that impair multimer formation, or that lead to excessive cleavage of multimers by ADAMTS13, cause a bleeding disorder (von Willebrand disease, discussed further in Chapter 12). The bottom line is that **size matters for vWF adhesive function.**

C. Platelet Function

1. Platelet adhesion

vWF binds to exposed extracellular matrix following injury, and unfolds. This exposes binding sites for platelet glycoprotein Ib (Gp Ib). High shear created by flowing blood promotes unfolding of the vWF multimer, which allows binding to platelets. Thus, vWF that has bound to the extracellular matrix at the site of injury and has unfolded binds avidly to platelets, whereas circulating, folded vWF does not. vWF binding and platelet adhesion also serve to localize coagulation factor VIII (bound to vWF) and factor V (in platelet α -granules) to the site of injury.

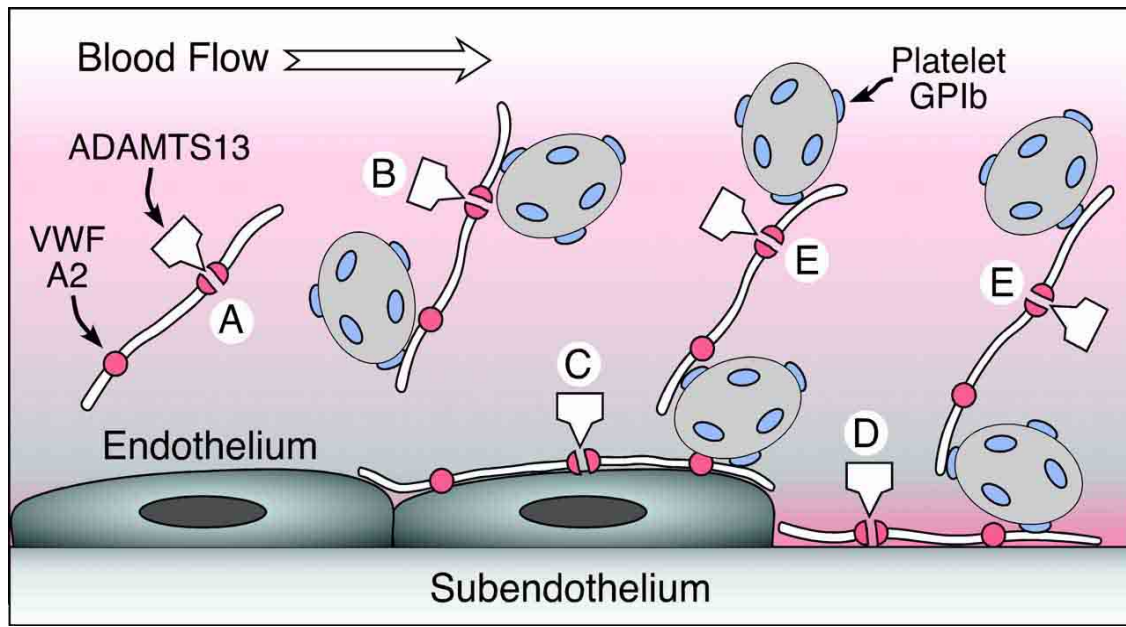


Figure 11.3- Cleavage of vWF by ADAMTS13. vWF is released from endothelial cells as very large multimers that may diffuse into the circulation (A and B) or adhere to the endothelial cell surface (C). vWF also binds to connective tissue exposed at sites of vascular injury (D). Under conditions of high fluid shear stress, platelets can adhere to vWF in solution (B) or on surfaces (C and D) through the platelet glycoprotein Ib (GPIb) receptor. vWF also may recruit platelets to previously adhering platelets (E). ADAMTS13 cleaves a specific bond in the vWF subunit, severing the multimer. This reaction is slow for vWF in solution (A) but occurs rapidly when platelets adhere to vWF under high fluid shear conditions in suspension (B) or on surfaces (C-E), presumably as a consequence of conformational changes induced by tensile force on the vWF multimer. Failure of this mechanism causes Thrombotic Thrombocytopenic Purpura (TTP, discussed in Chapter 12). (Figure from: Sadler, JE, *PNAS* 99:11552, 2002)

2. Platelet activation

Once platelets have become tethered to the subendothelial matrix by vWF, additional ligands interact with specific platelet receptors and trigger platelet activation. **Strong platelet agonists include collagen in the extracellular matrix and thrombin generated by tissue factor exposure at the site of injury.** Other platelet agonists include epinephrine, ADP, serotonin, and thromboxane A₂. **Thromboxane A₂ produced from platelet arachidonic acid via the aspirin-sensitive cyclo-oxygenase pathway (COX-1) diffuses into the blood and activates additional platelets.**

Platelet activation is a complex process in which agonists stimulate platelet shape change and spreading (see Figure 11.2), conformational activation of glycoprotein IIb/IIIa (Gp IIb/IIIa), and binding sites for coagulation proteins. This process is also linked to subsequent platelet degranulation and clot retraction.

3. Platelet aggregation (platelet-platelet interaction)

Rapid formation of platelet aggregates at the site of injury helps to “plug the hole” and minimize blood loss. **The key regulatory event in the formation of platelet aggregates is a conformational change in the GpIIb/IIIa receptor that accompanies platelet activation. This conformational change allows the receptor to bind**

fibrinogen, which “glues” the platelets together. Circulating non-activated platelets cannot bind to fibrinogen, so that platelet aggregation is limited to sites of injury.

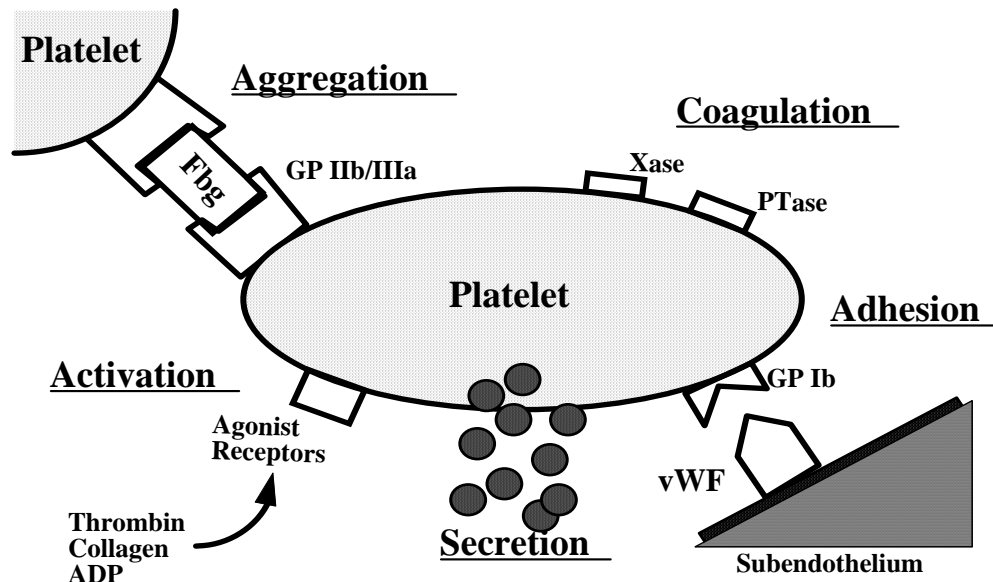


Figure 11.4. Components of the platelet response. Include the initial interaction of platelets with non-endothelial surfaces (adhesion), agonist stimulation via specific receptors (activation), platelet-platelet interaction (aggregation), release of granule contents (secretion), and formation of coagulation enzyme complexes on the platelet surface (procoagulant effect). See text for details. GP Ib = platelet glycoprotein Ib-V-IX complex, GP IIb/IIIa = platelet glycoprotein IIb/IIIa complex, Fbg- fibrinogen, vWF- von Willebrand factor, Xase and PTase- IXa/VIIIa (sometimes called “tenase” because it cleaves and activates factor X) and Xa/Va (called prothrombinase for a similar reason) enzyme complexes, respectively (see discussion of coagulation cascade).

4. Platelet secretion (Degranulation)

Activation of platelets by strong agonists results in secretion of both α -granule and dense granule contents that locally enhance coagulation, platelet adhesion and activation, vasoconstriction, and wound repair. In particular, α -granules release adhesive proteins such as fibrinogen and vWF and enhance coagulation via release of factor V. Dense granules release ADP/ATP and serotonin, which activate additional platelets. Growth factor and chemokine release by the platelet help to recruit inflammatory cells and initiate wound healing.

5. Platelet procoagulant activity

Activated platelets provide a procoagulant surface that markedly accelerates local thrombin generation. This procoagulant activity results, in large part, from exposure of phosphatidylserine (PS) on the outer membrane. PS is normally sequestered on the inner membrane leaflet of the platelet; it is exposed after platelet activation. Exposure of PS enhances the assembly of the membrane-bound coagulation enzyme complexes, including factor IXa-factor VIIIa and factor Xa-factor Va (see Figure 11.6).

Summary of the events that occur when a blood vessel is injured:

1. **Vasoconstriction:** Slows the leak and brings all of the necessary elements needed to plug the hole into the vicinity.
2. **A platelet plug forms** within 2-3 minutes.
3. **An insoluble fibrin mesh is deposited around the platelet plug** in about 5 minutes.
4. Bleeding stops.

Summary of the events involved in platelet plug formation:

1. Platelets **adhere** to the subendothelium at the site of injury. This is mediated by von Willebrand factor.
2. Platelets **spread** to cover the site of injury.
3. Platelets become **activated**, causing them to expose receptors for coagulation proteins and release substances that recruit more platelets to the site of injury.
4. Platelets **aggregate**. Fibrinogen binds to a receptor on the platelet surface and sticks platelets together.

III. Secondary Hemostasis: Coagulation and Fibrin Clot Formation

A. Coagulation Cascade-

The coagulation cascade is a series of reactions in which the sequential activation of serine proteases generates thrombin (factor IIa), the enzyme that converts soluble fibrinogen to an insoluble fibrin clot. The fibrin clot is deposited over and around the platelet plug, securing it firmly in place (much like the wire cage around the cork in a champagne bottle). The coagulation factors (enzyme or cofactor) are identified by roman numerals. A small “a” following the numeral indicates the activated form. Historically, this cascade has been viewed as two separate arms, the extrinsic and intrinsic pathways, which correlate with widely used screening tests for coagulation (Figure 11.5). The extrinsic pathway is a term given to the clotting of plasma in response to addition of tissue homogenates (i.e., requiring factors “extrinsic” to the blood), as measured by the **prothrombin time (PT)**. The intrinsic pathway refers to the clotting of plasma placed in a glass tube (requiring only “intrinsic” blood factors), as measured by the **activated partial thromboplastin time (aPTT)**. The PT and aPTT are discussed in more detail below. While extrinsic and intrinsic pathways are useful concepts for laboratory testing, they do **not** describe the *in vivo* coagulation response. **The cascade is actually a single pathway with two major stages: an initiation phase triggered by formation of the tissue factor (TF)-factor VIIa complex, and a propagation phase, which amplifies the response through the factor IXa-factor VIIIa complex** (Figure 11.7). The initiation phase is a threshold-mediated event – that is, tissue factor-VIIa activity must exceed a certain threshold to trigger the clotting mechanism. Once this occurs, the propagation phase produces an exponential burst of thrombin that is responsible for fibrin clot formation.

1. Structure-function aspects of the membrane bound coagulation enzyme complexes

The coagulation factors can be grouped by function and structure into either proteases or cofactors. The vitamin K-dependent proteases include protein C, factor VIIa, factor IXa, factor Xa, and prothrombin (factor II). These proteins possess a γ -carboxylation (Gla) domain that mediates calcium-dependent binding to the membrane surface (Figure 11.6) and a serine protease domain that possesses enzymatic activity. These proteins are synthesized as proteolytically inactive zymogens by the liver, and undergo vitamin K-dependent post-translational γ -carboxylation of specific glutamic acid residues in the Gla domain. These γ -carboxylated glutamic acid residues bind calcium with high affinity. Calcium binding generates the proper conformation for subsequent membrane binding of the protease. Membrane binding is a critical step that brings a protease, a cofactor (helper protein), and a zymogen into close proximity (Figure 11.6). **Inhibition of vitamin K-dependent γ -carboxylation is the mechanism of action of the anticoagulant drug warfarin** (see Chapter 11, part 2). Zymogens are converted to active coagulation proteases by limited proteolytic cleavage. Factor XI is a structurally related serine protease but does **not** contain a Gla domain (and therefore is not “vitamin K-dependent”). Factor XIII is a structurally unrelated enzyme that is activated by thrombin and crosslinks fibrin to stabilize the thrombus (see below).

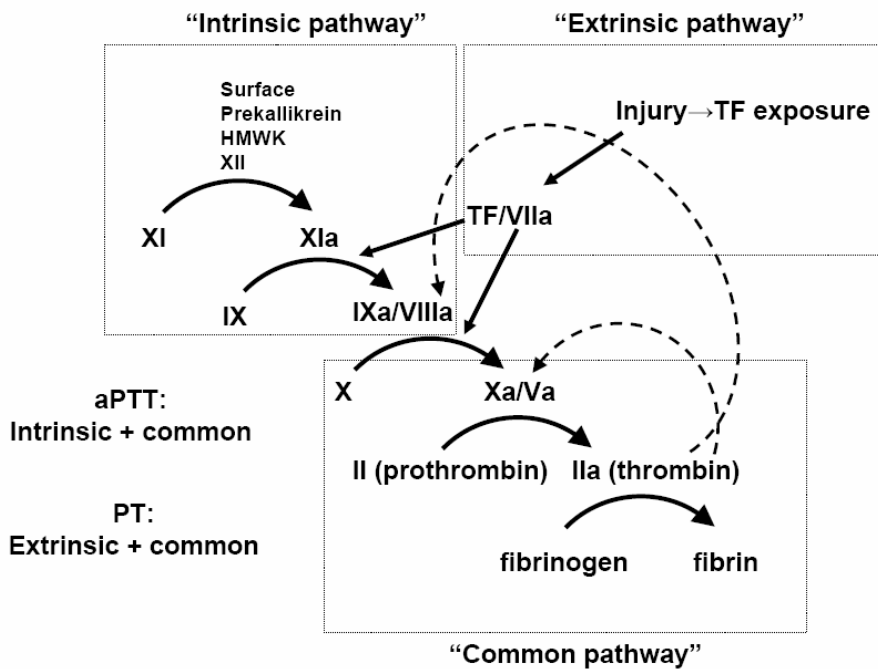


Figure 11.5- “Extrinsic” and “intrinsic” coagulation pathways. These pathways highlight the factors required for two useful diagnostic tests, the prothrombin time (PT) and activated partial thromboplastin time (aPTT), respectively. A substance “extrinsic” to blood that contains tissue factor (tissue homogenates or thromboplastin) triggers blood coagulation by the extrinsic pathway. This pathway requires the factors in the extrinsic and common regions (TF, VII, V, X, prothrombin, fibrinogen). Factors “intrinsic” to blood placed in a glass tube trigger coagulation by contact activation in the intrinsic pathway. This pathway requires the factors involved in the intrinsic and common regions (PK, HMWK, XII, XI, VIII, IX, V, X, prothrombin, fibrinogen). The extrinsic pathway correlates with the initiation phase, and some of the components of the intrinsic pathway contribute to the propagation phase. The “contact activation” factors (PK, HMWK, fXII) have little if any role in physiologic coagulation. Dotted lines represent feedback activation of cofactors by thrombin. Thrombin also activates factor XI (not shown).

FYI

Procoagulant and Anticoagulant Proteins

Procoagulant Proteins:

| Protein | Size (kD) | Concentration (mg/dl) | Type of Protein | Function (after activation) |
|--------------------------|-----------|-----------------------|-----------------|---|
| Fibrinogen (FI) | 340 | 300 | Structural | Polymerizes to form clot |
| Prothrombin (FII) | 72.5 | 10 | VKZ | Activated fibrinogen, V, VIII, XIII, XI, protein C, platelets |
| Factor V | 350 | 2 | Cofactor | Supports activation of II by Xa |
| Factor VII | 50 | 0.1 | VKZ | Activates IX and X |
| Factor VIII | 350 | 0.1 | Cofactor | Supports activation of X by IXa |
| Factor IX | 57 | 1 | VKZ | Activates X |
| Factor X | 59 | 1 | VKZ | Activates II |
| Factor XI | 160 | 0.5 | Zymogen | Activates IX |
| Factor XIII | 320 | 3 | Zymogen | Crosslinks fibrin, other proteins |

Zymogen: inactive precursor to active protease; VKZ: vitamin K-dependent zymogen; Cofactor: helper protein that dramatically stimulates protease activity

Anticoagulant Proteins:

| Protein | Size (kD) | Concentration (mg/dl) | Type of Protein | Function (after activation) |
|---|-----------|-----------------------|--------------------|---|
| Antithrombin | 340 | 300 | Protease inhibitor | Inhibits thrombin (IIa), Xa, IXa, XIa |
| Protein C | 72.5 | 10 | VKZ | Proteolytic inactivation of V and VIII |
| Protein S | 350 | 2 | VK Cofactor | Supports inactivation of V and VIII by activated PC (APC) |
| Tissue factor pathway inhibitor (TFPI) | 50 | 0.1 | Protease inhibitor | Forms complex with Xa that inactivates VIIa/tissue factor complex |

VK cofactor: vitamin K-dependent cofactor

The cofactors are a more diverse group that includes protein S, tissue factor, factor V, and factor VIII. In basic terms, cofactors help bring a protease together with its substrate, thereby enhancing its proteolytic activity. Protein S is a vitamin K-dependent protein (possesses a Gla domain) that serves as a cofactor for the anticoagulant activated protein C (Figure 11.11). Tissue factor is an integral membrane protein that binds factor VIIa to form an enzyme complex that activates factor X. Protein S and tissue factor do **not** require proteolytic activation for cofactor activity. In contrast, factors V and VIII are large (>350,000 daltons) proteins that require proteolytic activation by thrombin to generate cofactor activity. Factor VIIIa is an unstable cofactor (activity degrades over time) that binds factor IXa to form a complex that activates factor X. Factor Va is a stable cofactor that binds factor Xa to form a complex that activates prothrombin.

Formation of membrane-bound complexes is critical to localization of procoagulant and anticoagulant activities. The component parts include: a serine protease (enzyme), a protein cofactor, calcium, an “activated” membrane surface, and a protein substrate (Figure 11.6). Activation of the membrane surface refers to exposure of negatively charged phospholipids such as phosphatidylserine (PS) on the outer membrane leaflet, which is required for the formation of these high affinity complexes. PS is normally sequestered on the inner membrane leaflet, but becomes exposed on the platelet surface after activation, or on other cells types during apoptosis or necrosis. Exposure of negatively charged PS promotes the calcium-dependent binding of vitamin-K dependent proteases and zymogens to the membrane. Factors Va and VIIIa bind to specific membrane receptors that are exposed during platelet activation. **The physiologic importance of the assembly of the factor IXa/VIIIa and factor Xa/Va complexes on the platelet surface cannot be overemphasized, as it enhances the catalytic efficiency of the proteases involved by 10,000 to 20,000-fold. No physiologically meaningful coagulation occurs in the absence of these fully formed enzyme complexes.**

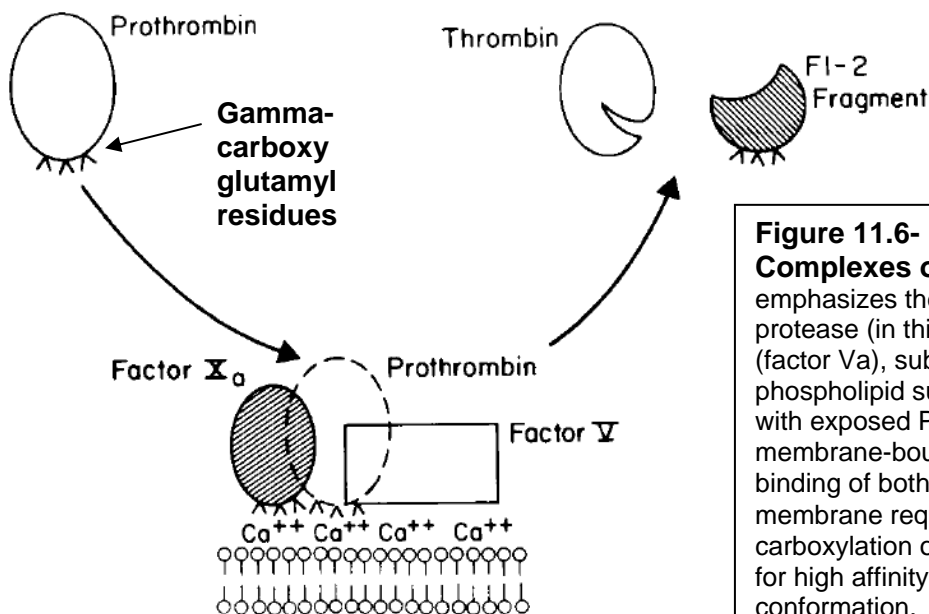


Figure 11.6- Membrane-Bound Enzyme Complexes of Coagulation. This cartoon emphasizes the relationships between protease (in this case factor Xa), cofactor (factor Va), substrate (prothrombin) and the phospholipid surface (activated membrane with exposed PS) during formation of the membrane-bound enzyme complexes. The binding of both protease and zymogen to the membrane requires vitamin-K-dependent γ -carboxylation of their respective Gla domains, for high affinity calcium binding and proper conformation. Unbound proteases have dramatically lower catalytic activity, thereby localizing coagulation reactions to the membrane surface.

2. Initiation phase: Tissue factor exposure leads to generation of small amounts of thrombin.

Tissue factor (TF) is an integral membrane protein expressed on the surface of a variety of cell types normally located outside the vasculature. Following vascular injury, TF is exposed to the blood and binds the protease factor VIIa (1-2% of the total factor VII circulates as factor VIIa). During this initiation phase, the TF-factor VIIa complex activates additional factor VII (to VIIa), and small amounts of factors IX and X (<1% of total zymogen) before being inhibited by tissue factor pathway inhibitor (see below). Factor Xa converts a small amount of prothrombin to thrombin, which then activates the cofactors, factors V and VIII, and the protease factor XI. This cofactor activation is critical for formation of the membrane-bound enzyme complexes required for the propagation phase.

3. Propagation phase: Thrombin generation is amplified through activation of cofactors VIII and V and factor XI.

Activation of the cofactors, factors VIII and V, allows formation of the membrane bound enzyme complexes, IXa/VIIIa and Xa/Va (Fig 11.7). TF-factor VIIa activity has to exceed a threshold level to trigger propagation (i.e., activate cofactors), but has relatively little effect on the final rate of thrombin generation. In contrast, the IXa/ VIIIa complex is required for the explosive generation of thrombin from prothrombin that occurs in the propagation phase. Thrombin also cleaves factor XI to XIa, which activates additional factor IX, and contributes to the final rate of thrombin generation. **Thus, the TF-factor VIIa pathway is a threshold-mediated trigger (initiation phase), and the factor IXa-factor VIIIa pathway markedly amplifies the coagulation response (propagation phase), resulting in an explosive increase in thrombin generation.**

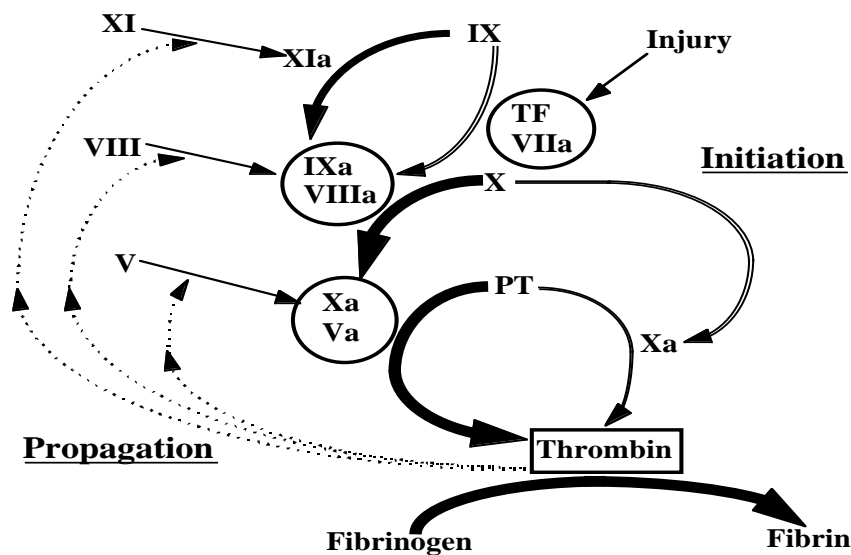


Figure 11.7- Schematic display of the *in vivo* coagulation cascade. The coagulation factors are represented by Roman numerals except for tissue factor (TF), prothrombin (PT), and fibrinogen, the suffix "a" indicates the activated form of the protease (factors VIIa, IXa, Xa, XIa) or cofactor (factors Va and VIIIa), and the ovals represent membrane-bound coagulation complexes. The initiation phase (light, double-lined arrows) is mediated by the TF-VIIa complex. Dotted arrows show thrombin-catalyzed activation of cofactors V and VIII and factor XI. The propagation phase is mediated by the VIIIa-IXa and Va-Xa complexes (solid arrows).

complex changes the substrate specificity of thrombin from pro-coagulant to anticoagulant, accelerating the rate of protein C activation (see circulating coagulation inhibitors, below). In addition to activation of platelets, thrombin also can activate related surface receptors on additional cell types including endothelium, monocytes, smooth muscle cells, and fibroblasts. These activities modulate the migration of inflammatory cells, wound healing, and a variety of pathologic processes including intimal proliferation, atheroma formation, and tissue remodeling/fibrosis.

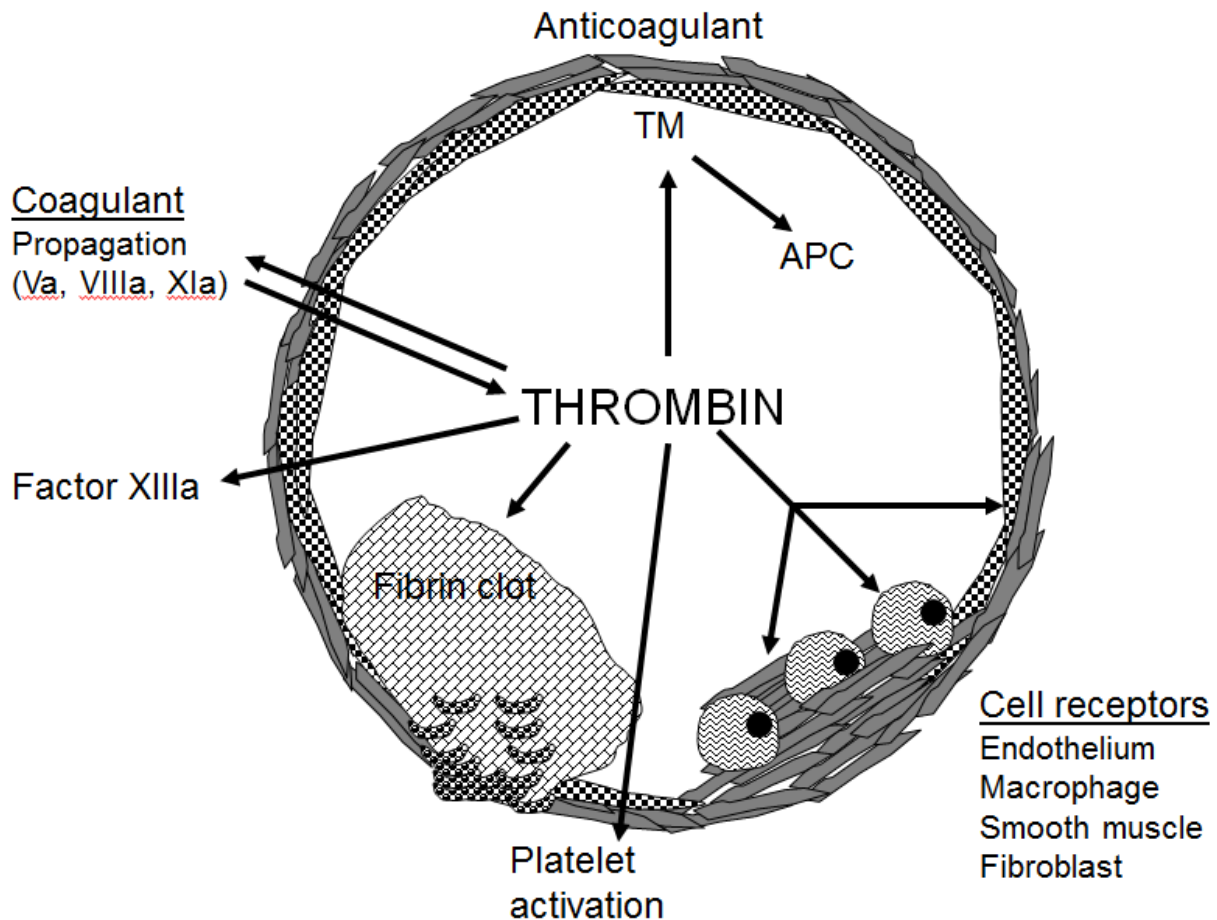


Figure 11.9- The central role of thrombin in vascular injury. See text for details. TM- thrombomodulin, APC- activated protein C.

6. Contact activation system

Individuals with factor XII, high molecular weight kininogen (HMWK), or prekallikrein deficiency do not exhibit a bleeding phenotype, indicating that these factors are not required for normal hemostasis. Clinically, the most important aspect of these proteins is that they are required for a normal result in the activated partial thromboplastin time (aPTT). Thus, in patients with a prolonged aPTT, one needs to differentiate between deficiencies of hemostatically important factors versus the contact activation factors.

7. The role of factor XI

In contrast to the contact activation factors, factor XI deficiency demonstrates a clinical bleeding phenotype, although less severe than hemophilia A or B (factor VIII or IX deficiency). Factor XI is activated by thrombin on the platelet surface, and factor XIa then activates factor IX.

Summary of the steps in fibrin clot formation

1. Injury exposes the subendothelium of the blood vessel and tissue factor is exposed to the blood.
2. Tissue factor combines with factor VIIa, and this complex activates a small amount of factor X. This initiates the clotting cascade and forms a small amount of thrombin.
3. Thrombin explosively propagates coagulation by activating the cofactor proteins V and VIII, and factor XI. Much more thrombin is formed.
4. Thrombin converts fibrinogen to fibrin, which polymerizes to form the clot.
5. Thrombin activates factor XIII, which crosslinks and stabilizes the clot.
6. Clotting is localized to the platelet plug, because the vitamin K-dependent proteases and their substrate zymogens bind avidly to phospholipids exposed when platelets are activated, and factors Va and VIIIa bind to specific receptors exposed by platelet activation.

You are now ready to consider how clot formation is quickly shut down after the hole in the vessel is plugged. This prevents the clot from extending beyond the site of injury.

B. Circulating Coagulation Inhibitors

The coagulation cascade is tightly regulated by three major anticoagulant systems: tissue factor pathway inhibitor (TFPI), antithrombin III (AT3), and activated protein C (APC) (Figure 11.10). AT3 is also referred to as antithrombin or AT. TFPI inhibits the tissue factor-factor VIIa complex, thus regulating the initiation phase. AT3 also has a modest effect on the initiation phase, but markedly reduces the rate of thrombin generation in the propagation phase, and also inhibits thrombin itself. The combination of TFPI and AT3 is able to suppress thrombin generation at low TF-factor VIIa concentrations. Activated protein C (APC) inactivates the active cofactors Va and VIIIa by proteolysis, thereby terminating the propagation phase of coagulation.

3. Activated protein C pathway (Figure 11.11)

Protein C is a vitamin K-dependent zymogen, homologous to the procoagulant factors VII, IX, and X. Unlike those factors, it serves an anticoagulant function. **Activated protein C (APC) inactivates factors VIIIa and Va by proteolysis, which terminates the propagation phase of coagulation. Protein S is a non-enzymatic cofactor for APC**, and is especially important for inactivation of factors VIIIa and Va within their respective membrane-bound complexes. Protein C (zymogen) is activated by thrombin bound to the endothelial surface protein thrombomodulin. Formation of the high affinity thrombin-thrombomodulin complex inhibits the pro-coagulant functions of thrombin, and markedly accelerates the activation of PC. **The clinical importance of the protein C pathway is emphasized by the increased risk of thrombosis associated with inherited protein C and S deficiencies and factor V Leiden (FVL). FVL is a protein C-resistant form of factor Va due to mutation of one of the APC cleavage sites.** The activated protein C pathway does not affect the initiation phase of coagulation, but does regulate and terminate thrombin generation during the propagation phase.

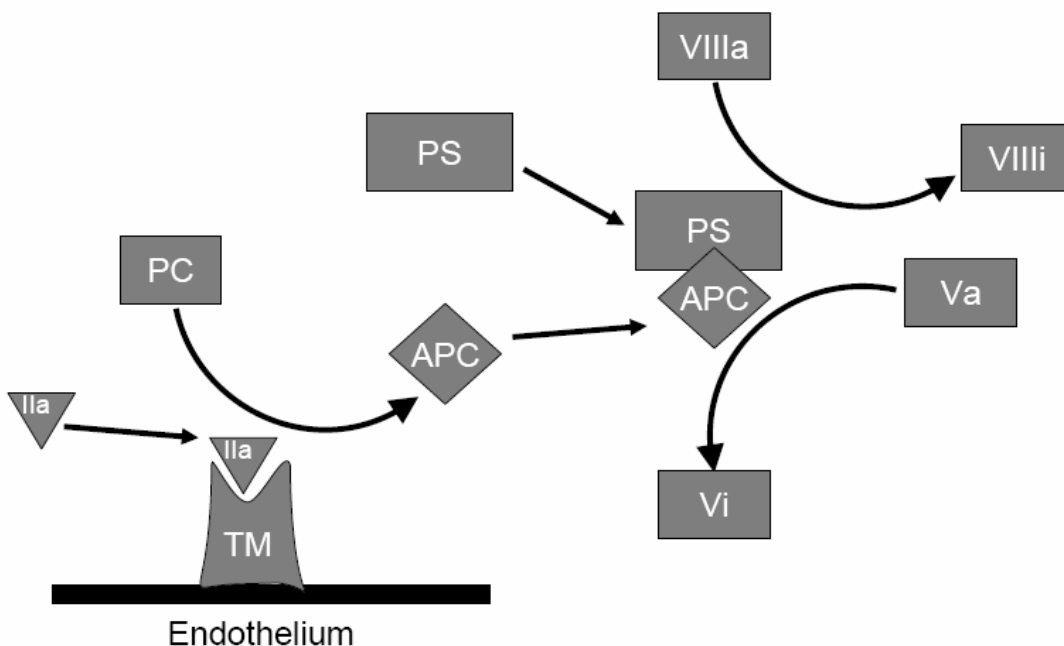


Figure 11.11- Thrombomodulin-protein C anticoagulant pathway. Thrombin (IIa) bound to thrombomodulin (TM) accelerates the activation of PC (zymogen) to APC (protease). APC combines with the cofactor protein S. The APC-PS complex proteolytically inactivates factors Va and VIIIa. *IIa- thrombin, TM- thrombomodulin, PC- protein C, APC- activated protein C, PS- protein S, Vi- inactivated factor V, and VIIIi- inactivated factor VIII.*

5. Regulation of coagulation: The gun analogy

The coagulation response can be likened to a gun aimed at the site of vascular injury. Exposure of tissue factor by injury results in formation of the factor VIIa-tissue factor complex, which is the “trigger” for coagulation, and is down-regulated by TFPI and AT3.

Initiation is a threshold-mediated event, meaning that the “trigger” has to be squeezed hard enough for the gun to go off, which requires sufficient thrombin generation to activate the cofactors (factors V and VIII). Some stimuli will be inadequate and promptly shut down by TFPI. However, once the threshold is exceeded and cofactor activation has occurred, the propagation phase is activated and the gun is “fired”. The propagation phase requires assembly of the membrane-bound IXa/VIIIa and Xa/Va complexes. The propagation phase results in an exponential increase in thrombin generation at the site of injury, and formation of the fibrin clot. The response is terminated by inactivation of VIIIa and Va by APC, and inhibition of thrombin, Xa, and IXa by AT3.

C. Integration of the Platelet and Coagulation Responses

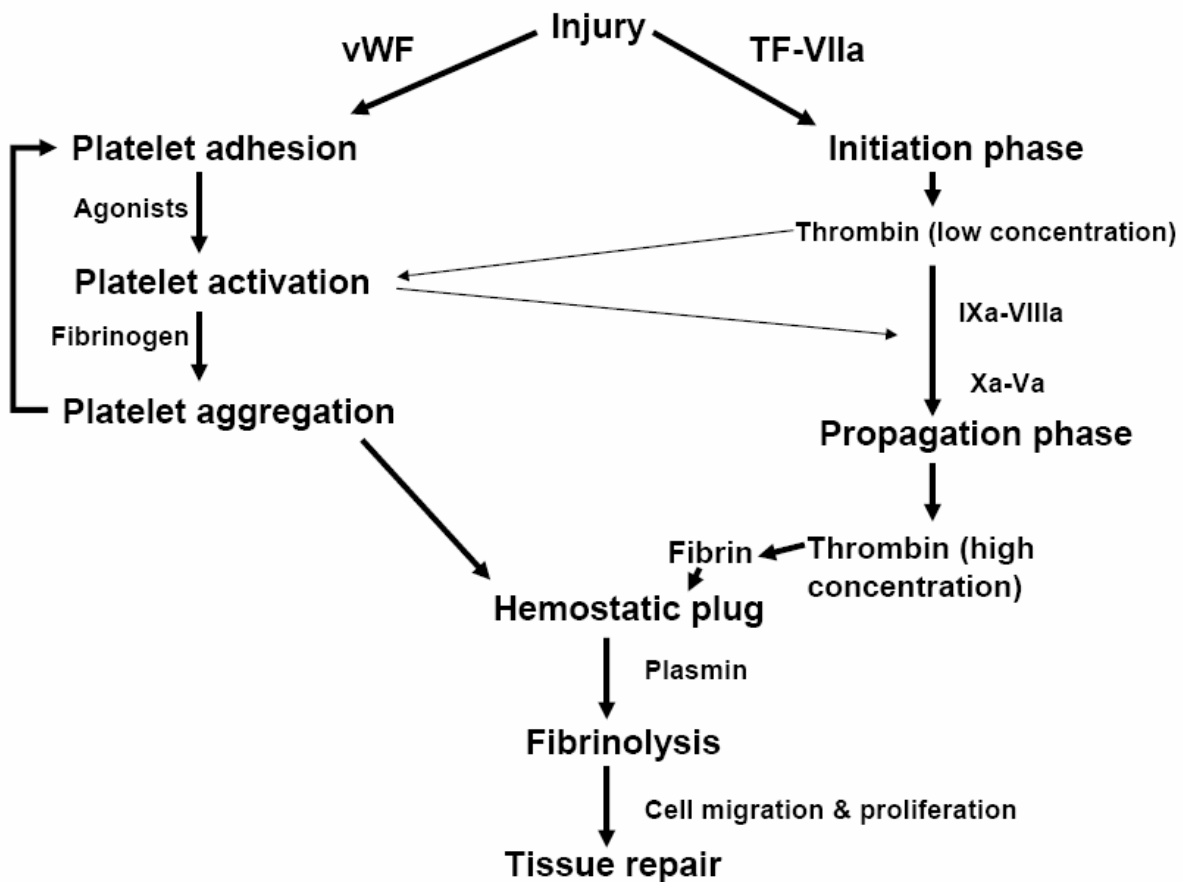


Figure 11.12- Integration of pro-coagulant arms of hemostatic response. The platelet and coagulant responses are highly integrated. Picomolar (10^{-12} M) amounts of thrombin generated by the initiation phase of coagulation are sufficient to activate platelets locally, which then expose membrane phosphatidylserine (PS) and provide an important surface for assembly of the IXa/VIIIa and Xa/Va complexes. Likewise, the binding of vWF and release of platelet granule contents increase local concentrations of factors VIII and V, respectively. The platelet surface also facilitates factor XI activation by thrombin, generating additional factor IXa and enhancing the propagation phase response. Although the relative contribution of platelet and coagulant responses varies between the arterial and venous circulation, they are never completely independent.

While the platelet and coagulation responses have been discussed as separate events, they are fully integrated *in vivo* (Figure 11.12). In general, **the platelet response predominates on the arterial side, and the coagulant response is more critical on the venous side. However, there is always involvement of both components.**

IV. Antithrombotic Components

A. Endothelium

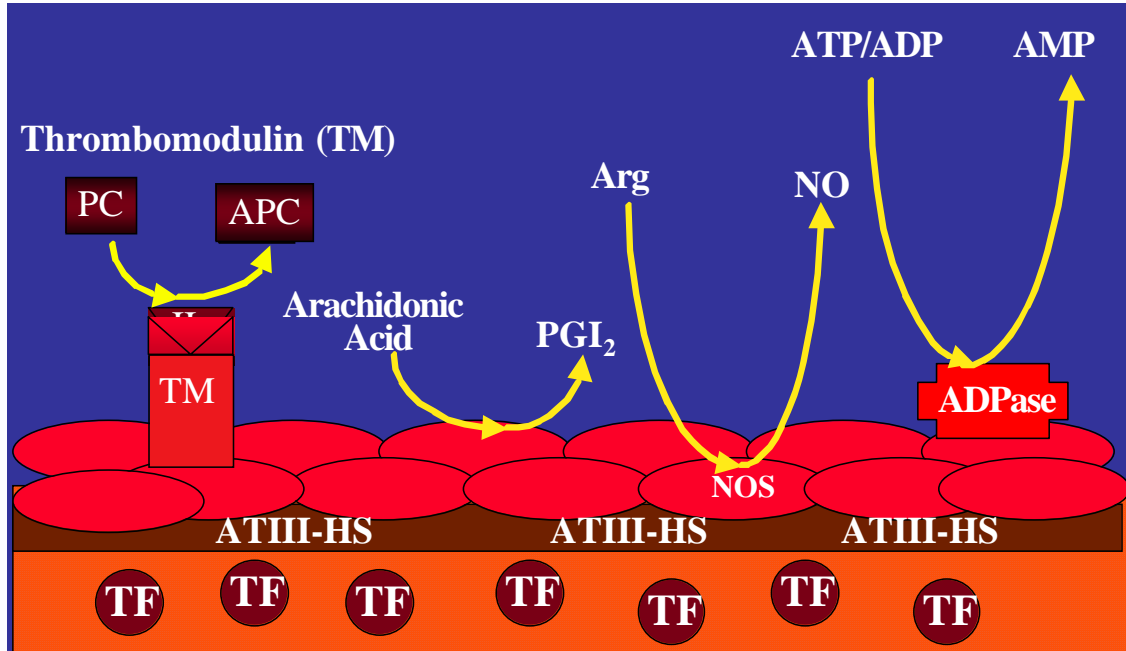


Figure 11.13- Antithrombotic functions of endothelium. See text for discussion. IIa- thrombin, PC- protein C, APC-activated protein C, TM- thrombomodulin, PGI₂- prostacyclin, Arg- arginine, NO- nitric oxide, NOS- nitric oxide synthetase, AT3-HS- antithrombin III-heparan sulfate complex, TF- tissue factor.

Antithrombotic Properties

The endothelium provides a non-thrombogenic surface that regulates platelet and coagulation responses (Figure 11.13). When undisturbed, the endothelial surface possesses anti-platelet, anticoagulant, and pro-fibrinolytic properties. It also provides an important barrier function, sequestering active tissue factor from the circulating blood and regulating the egress of inflammatory cells and fluids. The platelet inhibitory properties of the endothelium include expression of prostacyclin (PGI₂), endothelial nitric oxide synthase (eNOS), and the ectoADPase (CD39). Prostacyclin produced via the arachidonic acid pathway opposes the activity of platelet thromboxane A₂ at the endothelial surface. PGI₂ causes vasodilatation and inhibits platelet activation. NO is generated from arginine by eNOS, resulting in vasodilatation and platelet inhibition. ADPase is expressed at the endothelial surface and enzymatically degrades ADP and ATP, thereby inhibiting platelet activation and recruitment. Anticoagulant properties of the endothelium include acting as a barrier to tissue factor exposure, heparan sulfate expression on

the cell surface and in the subendothelial matrix, and cell surface expression of thrombomodulin. Sequestration of tissue factor by the endothelial barrier prevents triggering of coagulation under normal conditions. Heparan sulfate binds the inhibitor AT3 to accelerate protease inhibition on the endothelial surface. Likewise, expression of thrombomodulin markedly enhances the generation of activated protein C at low thrombin concentrations. Finally, the endothelial surface is profibrinolytic due to the expression of surface receptors for these components (see below). Thus, **under normal circumstances, thrombogenesis is suppressed by multiple negative regulators at the endothelial surface.**

FYI

Heterogeneity of Endothelium:

The properties of the endothelial barrier are rather heterogeneous and appear to be significantly influenced by the tissue in which the vessels develop. The clearest example of this specialization is the endothelial blood-brain barrier. Likewise, stimulation of endothelium by inflammatory cytokines such as TNF in pathologic situations can down-regulate the anti-thrombotic mechanisms, induce tissue factor expression, and predispose towards the development of thrombosis. The properties of endothelium undergo a profound change when stimulated to undergo angiogenesis, the development of new vessels from pre-existing vessels. These differences are of potential therapeutic interest in the repair of ischemic injury, and in the targeting of blood vessel formation during growth and metastasis of malignancies.

Summary – regulation of coagulation:

1. **How unwanted clotting is prevented:** Normal uninjured endothelium actively inhibits clotting by a variety of mechanisms outlined above.
2. **How clotting is confined to the site of injury:**
 - a. Activated clotting factors are localized to the surface of the platelet plug.
 - b. Antithrombin III inactivates any thrombin that escapes into the circulation
 - c. Thrombin binds to the endothelial surface, where it is transformed from a procoagulant to an anticoagulant by binding to thrombomodulin. The thrombin thrombomodulin complex activates protein C, and activated protein C inactivates factors Va and VIIIa. Clot propagation is shut down and clot extension is prevented.

You are now ready to consider how the wound heals and the clot resolves.

B. Fibrinolysis

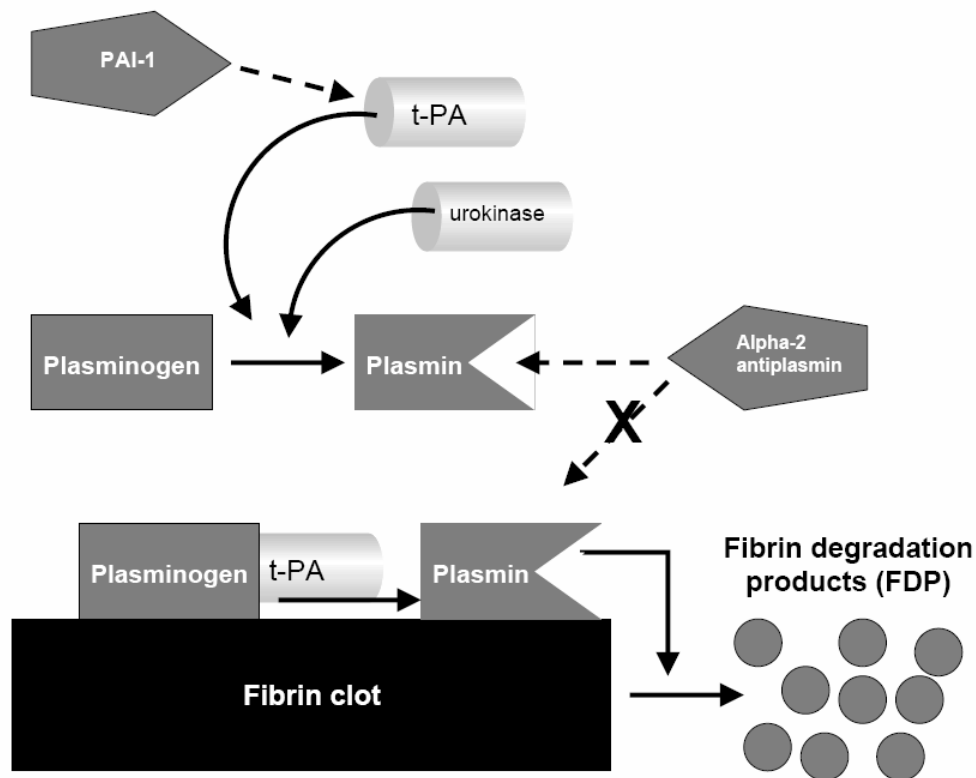


Figure 11.14- Fibrinolytic system. Plasminogen activators include the relatively fibrin-specific tPA, and non-specific urokinase. Plasminogen activator inhibitor 1 (PAI-1) inhibits the plasminogen activators. Activation of plasminogen by tPA is enhanced when both are bound to fibrin, and inhibition of free plasmin by α_2 -antiplasmin is much more efficient in the fluid phase, encouraging localization of plasmin activity to the fibrin clot. Dashed lines indicate an inhibitory effect.

The fibrinolytic pathway is a relatively simple serine protease cascade that promotes breakdown of fibrin clots (Figure 11.14). The ultimate product of this pathway is plasmin, a serine protease that digests the fibrin clot. Plasmin activity is regulated by protease inhibitors, localization to fibrin, and endothelial surface receptors. The fibrinolytic pathway helps to remove fibrin deposits that may form inappropriately in normal vessels, remodels thrombus after vascular injury, and participates in wound repair. The major components include:

1. Plasminogen/plasmin

Plasmin is the primary product of the fibrinolytic pathway and is the protease responsible for degrading fibrin. Plasminogen is synthesized in the liver as a zymogen that circulates with a long half-life (2 days). Plasminogen is converted to an active protease by plasminogen activators (see below). **Digestion of cross-linked fibrin by plasmin generates a specific plasmin degradation product called D-Dimer, which is important clinically in the diagnosis of disseminated intravascular coagulation (DIC) and venous thromboembolism** (see Chapters 12 and 13) (Figure 11.15). In pathologic situations, excess circulating plasmin may cause bleeding by degradation of factors V, VIII, and fibrinogen, and by causing rapid breakdown of clots. However,

similar to the localization of thrombin generation during coagulation, plasmin generation during fibrinolysis is normally restricted to the fibrin clot or endothelial surface.

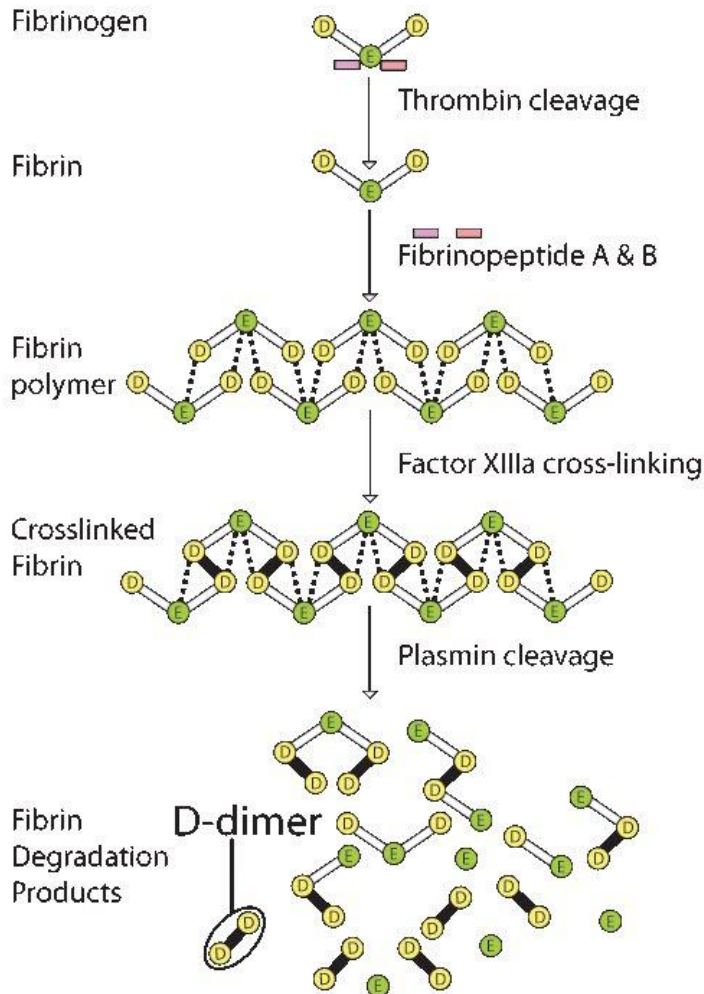


Figure 11.15. Generation of D-dimer from cross-linked fibrin. Source: <https://ahdc.vet.cornell.edu/Sects/Coag/test/Ddimer.cfm>

2. Plasminogen activators

Endothelial cells secrete the plasminogen activators tissue plasminogen activator (tPA) and urokinase, which proteolytically activate plasminogen to plasmin. tPA binds specifically to fibrin. Thus, fibrin acts as a cofactor for tPA activation of plasminogen, and so helps catalyze its own destruction. In contrast, urokinase has relatively low affinity for fibrin, and activates plasminogen equally well in the presence or absence of fibrin.

Cellular receptors for both plasminogen activators and plasmin(ogen) maintain a profibrinolytic endothelial surface, which helps to remove fibrin that forms in places where it is not needed. Plasminogen and tPA bind to receptors on the endothelial cell surface, which stimulate plasminogen activation approximately 60-fold.

3. Fibrinolysis Inhibitors. As is the case with coagulation, proper balance between pro- and anti-fibrinolytic processes is necessary to maintain homeostasis. The most important antifibrinolytic factors include:

- **α_2 -antiplasmin (α_2 -PI)** is synthesized in the liver and present at relatively high plasma concentrations with a long half-life (2.4 days). It rapidly inhibits circulating plasmin by forming a 1:1 complex, but is less efficient at inhibiting plasmin bound to fibrin clot. Thus, similar to the effect of AT3 on coagulation proteases, α_2 -antiplasmin keeps plasmin activity localized to fibrin clot under normal conditions and avoids systemic fibrinolysis. Some α_2 -antiplasmin is also incorporated into fibrin clot itself, and may play a role in protecting the clot from premature fibrinolysis. Genetic or acquired (see bleeding section) α_2 -antiplasmin deficiency results in an increased bleeding risk.
- **Plasminogen activator inhibitor-1 (PAI-1)** is the major plasminogen activator inhibitor in human plasma. PAI-1 is released by endothelial cells, monocytes/macrophages, hepatocytes, adipocytes, and platelets. Platelet-derived PAI-1 may help stabilize blood clots by delaying the onset of fibrinolysis.

Final summary:

- To maintain proper homeostasis, coagulation must occur precisely where and when it is needed, i.e., at sites of injury.
- This requires a delicate balance between factors that promote clotting and factors that inhibit clotting and break down clots.
- This is a complex system and it often goes awry. If any portion of the clotting system does not function properly, excessive bleeding may result. Bleeding disorders are discussed in Chapter 12.
- If clotting gets the upper hand venous thrombosis, pulmonary embolism, heart attack or stroke may result. Thrombotic disorders are discussed in Chapter 13.

V. Laboratory Testing

A. Tests used to assess the coagulation cascade:

- **Prothrombin time (PT)** measures factors involved in the initiation phase of coagulation (the “extrinsic pathway”), and is sensitive to **factors VII, X, V, prothrombin, and fibrinogen**. Selective prolongation of the PT (with a normal aPTT) occurs in factor VII deficiency. Deficiencies in the common pathway (factors X and V, prothrombin and fibrinogen) usually prolong both the PT and aPTT. The PT is not sensitive to deficiencies of factors VIII, IX or XI. To perform the PT, a thromboplastin reagent containing tissue factor, phospholipid, and calcium is added to citrated plasma, and the time required for fibrin clot formation is determined. **The prothrombin time is usually expressed the International Normalized Ratio or INR.** This is done to facilitate comparison of results from different laboratories,

specifically when monitoring treatment with the oral anticoagulant warfarin (see Chapter 11 part 2).

- **Activated partial thromboplastin time (aPTT)** measures factors involved in the propagation phase and contact activation (the “intrinsic pathway”), and is sensitive to prekallikrein*, high molecular weight kininogen*, factor XII*, **factors XI, IX, VIII, X, V, prothrombin, and fibrinogen. Deficiency of any of the contact activation factors (indicated by*) may prolong the aPTT but does not cause bleeding.** Selective prolongation of the aPTT (i.e., long aPTT with normal PT) occurs with deficiencies of the contact factors, or factors XI, IX, and VIII. To perform the aPTT, a reagent containing phospholipid plus a surface activator such as silica is pre-incubated with citrated plasma for 3 min. Plasma is then re-calcified and the time required for fibrin clot formation is determined.
- **Mixing tests- if a clotting time is prolonged, repeating the test with a 50/50 mix of patient plasma and normal plasma can assess whether the prolongation is due to clotting factor deficiency or a coagulation inhibitor** (see Chapter 12). Mixing tests are most often done to evaluate a markedly prolonged aPTT. Restoration of 30-40% normal levels of any coagulation factor is sufficient to correct the clotting time. Thus, if a single coagulation factor deficiency is present, the mixing test will completely correct prolongation of the clotting time. If a coagulation inhibitor is present, mixing will usually not correct the clotting time. An important caveat for interpretation of mixing studies is that a mixture of plasma with a prolonged clotting time due to major deficiency of multiple factors (warfarin, liver disease) with normal plasma may have a clotting time that is still a few seconds above normal .
- **Specific factor activity-** If the initial 50/50 mix with normal plasma completely corrects (suggesting a factor deficiency), the missing factor can be identified by mixing the patient’s plasma with plasma deficient in specific clotting factors whose deficiency might be responsible for the prolonged clotting time. For example, plasma from a patient with hemophilia A (factor VIII deficiency) will shorten clotting times when added to plasma deficient in any factor *except* factor VIII. The degree of deficiency (clotting factor activity) is quantified by measuring clotting times of serial dilutions of patient plasma into plasma that lacks the specific factor of interest, and comparing the results to those obtained from similar mixtures of normal plasma and factor-deficient plasma.

B. Platelets and vWF

1. **Platelet count-** A platelet count of 60,000-80,000/ μ l is adequate for normal hemostasis. Review of the peripheral smear is necessary to exclude platelet clumping as the cause of a low platelet count. Platelet morphology can help differentiate between peripheral destruction and bone marrow failure. A relative increase in platelet size (mean platelet volume) is often observed when marrow production is increased to compensate for peripheral destruction.

2. Platelet function testing:

- **Bleeding time-** is an older screening test of primary hemostasis (platelet plug formation). It is done by making a standardized incision in the skin and measuring the time required for bleeding to stop. The clinical use of the bleeding time has declined significantly because of poor reproducibility due to variation in technique, and patient-specific variables related to skin microanatomy. This test is not predictive of surgical bleeding.
- **PFA-100 (Platelet Function Analyzer-100) screen-** whole blood is passed through a small glass tube coated with collagen/epinephrine or collagen/ADP at high shear rates. The time until occlusion of the tube (closure time) is measured. This test is better standardized and less subject to problems due to variable technique than the bleeding time. However, like the bleeding time, it has not been shown to predict surgical bleeding. Abnormally long closure times may reflect abnormal platelet function (either inherited or acquired), a low platelet count, or low plasma VWF activity.
- **Platelet aggregation testing-** is a detailed assessment of platelet response (aggregation and secretion) to specific platelet agonists. It is used in selected patients with suspected intrinsic platelet defects, **not** as a screening test. Patient must pay fastidious attention to avoiding platelet inhibitory medications prior to testing.

3. vWF evaluation

- **vWF level-** antigenic level of the von Willebrand factor protein.
- **Factor VIII activity-** normally parallels vWF antigen (because factor VIII circulates in a complex with vWF).
- **vWF activity-** a variety of tests may be used to evaluate vWF function. Historically, **ristocetin cofactor activity** is the most commonly used test. Ristocetin is an antibiotic that induces a conformational change in vWF, triggering binding to platelet Gp Ib and in vitro platelet “agglutination”. Several other methods are now used as well.
- **Multimer analysis-** used in selected patients to evaluate the **size distribution** of vWF multimers. Selective loss of high molecular weight multimers results in decreased vWF function, and is seen in some variants of von Willebrand disease.

C. Fibrinolysis

- **FDP-** fibrin or fibrinogen degradation products (the products of fibrinolysis). This has largely been supplanted by the more specific D-dimer test, discussed below.
- **D-dimer-** a specific product that results from plasmin digestion of cross-linked fibrin (Figure 11.15). This test demonstrates the presence of both thrombin and plasmin activity (fibrin formation followed by proteolytic degradation of cross-linked fibrin, respectively) in the circulation. D-dimer and FDP levels generally rise in parallel.
- **Fibrinogen level.** The fibrinogen level may be low due to congenital deficiency of fibrinogen (rare), or to acquired deficiency due to conditions such as liver disease or disseminated intravascular coagulation (Chapter 12).

- **α_2 -antiplasmin level-** genetic deficiency represents a rare bleeding disorder, but more commonly low levels result from liver disease or from inhibitor consumption associated with circulating plasmin activity (e.g., disseminated intravascular coagulation – see Chapter 12).

4. Other useful tests

- **Thrombin Time (TT).** The test is performed by addition of thrombin to plasma and measuring the clotting time. The TT is sensitive to low fibrinogen levels and dysfibrinogenemias (dysfunctional fibrinogen – see below). It is also very sensitive to the presence of **heparin**.
- **Factor XIII activity-** screening test is urea clot solubility (non-cross-linked clot dissolves rapidly in 5 M urea). Confirmed by more specific assays. This test is mainly used to screen for congenital factor XIII deficiency, a very rare disorder.

CHAPTER 11

Part 2: ANTITHROMBOTIC AND HEMOSTATIC DRUGS

Key Concepts:

Pharmacology and clinical use of anticoagulant, thrombolytic, or antiplatelet drugs, as well as drugs that have hemostatic (anti-bleeding) effects.

Learning Objectives:

1. Compare and contrast unfractionated heparin, low molecular weight heparin, and fondaparinux in terms of structure, mechanism of action, and pharmacology.
2. Describe the indications, mechanism of action, appropriate laboratory monitoring, types of drug/food interactions, and management of overdosage for warfarin.
3. Compare and contrast warfarin, dabigatran, and rivaroxaban in terms of mechanism of action and pharmacology.
4. Identify which drug(s) would be best to give to a pregnant woman with deep vein thrombosis.
5. Compare and contrast the approach to treatment for a patient with arterial thrombosis to a patient with venous thrombosis
6. Describe how aspirin and clopidogrel work as antithrombotic agents.
7. Identify which antithrombotics have immediate effects and which ones take time to work, and why.
8. Describe the indications and mechanism of action for therapy with fibrinolytic agents, and list the important determinants of success with thrombolytic therapy.

Drug list:

Heparin

Low molecular weight heparins: Enoxaparin, dalteparin

Fondaparinux

Warfarin

Argatroban

Dabigatran

Rivaroxaban

Aspirin

Clopidogrel

Abciximab

Tissue plasminogen activator (alteplase, reteplase)

I. Overview of Pharmacologic Therapy

Antithrombotic therapy involves three major classes of drugs:

1. **Anticoagulants** – used to treat and/or prevent venous thrombosis and to prevent emboli from the heart
2. **Antiplatelet agents** – used to prevent arterial thrombosis
3. **Thrombolytic agents** – used when there is an acute need to reverse thrombosis

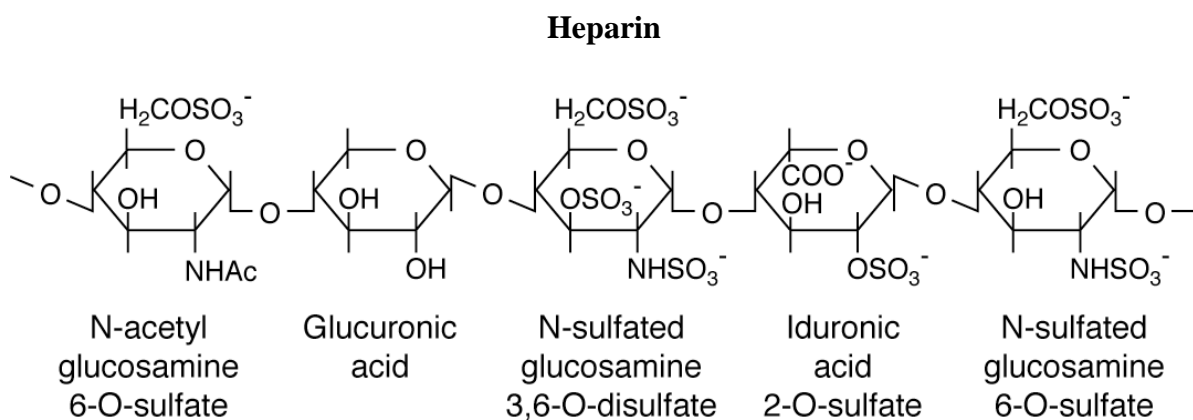
The cellular and molecular mechanisms in thrombosis are mostly the same as for normal hemostasis, with the main difference being the precipitating event and whether the clotting response is undesired and pathologic, or desired and normal. Thrombosis is treated with anticoagulant, anti-platelet, or thrombolytic drugs, but these drugs also interfere with normal hemostasis. *Therefore, bleeding is the most common undesired side effect of antithrombotic therapy.*

II. Anticoagulants:

A) Heparins

1. Background:

a) *Structure*- heparin is a heterodisperse mixture of negatively charged polysaccharide chains with a M_r range of 3,000 to 40,000 daltons. The basic unit is a repeating glucuronic/iduronic acid-glucosamine disaccharide which is heavily modified by O-sulfation (see figure below). Heparin is a naturally occurring component of mast cell secretory granules that is isolated from porcine intestinal mucosa or bovine lung tissue for therapeutic use.

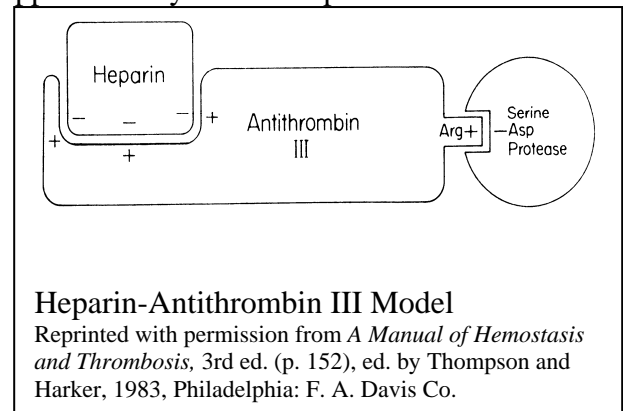


b) *Heparan sulfate (HS)*- is a naturally occurring glycosaminoglycan on the surface of cells and in the extracellular matrix. It possesses similar overall structure to heparin, except that sulfation is restricted to regularly spaced “islands” on the polymer. It also interacts with circulating

antithrombin III on the luminal surface of vascular endothelial cells providing a natural, local antithrombotic mechanism.

c) *Platelet factor 4*- is a chemokine released from platelet alpha granules during platelet activation. It binds to and neutralizes heparin, and thereby promotes local clot formation at the site of hemostasis.

2. Mechanism of action- the *anticoagulant effect* of heparin primarily results from the ability of this glycosaminoglycan to accelerate the inhibition of coagulation proteases (especially factor Xa and thrombin) by antithrombin (Figure 11.15). Heparin binds with high affinity to antithrombin via a specific pentasaccharide sequence present in approximately 1/3 of heparin chains. This interaction induces a conformational change in the inhibitor that accelerates the rate of protease inhibition. Heparin chain length influences target protease specificity (see comparison between UFH and LMWH below). [FYI: Heparin also has *anti-inflammatory properties* including inhibition of complement activation and selectin-dependent cell-cell interactions.]



3. Types of heparin

a) Unfractionated heparin (UFH)

Unfractionated heparin (UFH) is the naturally occurring form of heparin, and has been the primary drug for treatment of acute arterial and venous thrombosis since the 1940s. UFH has a MW range of 5,000-30,000, with 100% of the chains >18 saccharides. A chain length of >18 saccharides is required to accelerate thrombin inhibition by antithrombin, as that inhibition requires the binding of heparin to both antithrombin and thrombin simultaneously, whereas acceleration of factor Xa inhibition requires only binding to antithrombin (see Fig 11.15).

UFH is administered intravenously or subcutaneously, and is effective within minutes by the former route. It has variable and somewhat unpredictable pharmacokinetics, and so therapy is monitored using the aPTT, typically with a target of 1.5-2.5 times the control or baseline value. The PT/INR is usually not prolonged at these doses. Major complications are bleeding, heparin-induced thrombocytopenia (HIT—see Chapter 13), and (particularly with long-term use) accelerated osteoporosis, since heparin leaches calcium from bone. UFH can be neutralized *in vivo* by administration of protamine sulfate.

b) Low molecular weight heparin (LMWH)- *enoxaparin, dalteparin, others*

LMWH is a partially depolymerized heparin preparation (prepared by enzymatic or chemical degradation of UFH) with a MW range of 2,000-10,000 (average 5-6,000 Daltons). Because 50-75% of the chains are <18 saccharides, the effects of LMWH on factor Xa inhibition become more important, although 25-50% of the molecules can bind to, and therefore inhibit, thrombin. Thus, LMWH demonstrates an increased ratio of anti-factor Xa/anti-thrombin inhibitory activity. LMWH is usually administered subcutaneously once or twice daily (although it is possible to give intravenously), and does not reliably prolong the aPTT. Depletion of the

high molecular weight chains results in more predictable pharmacokinetics, allowing dosing by body weight without routine coagulation monitoring. LMWH is primarily cleared by the kidneys, with a plasma half-life of 4-6 hours. Thus, renal insufficiency is a relative contraindication for use. Treatment with LMWH appears to be equivalent to UH for prophylaxis and treatment of DVT/PE. LMWH appears to have a significantly lower incidence of HIT, and possibly osteoporosis, compared to UH. Minor chemical and size distribution differences exist between available LMWH products, but there is no convincing evidence for clinically important differences between these preparations. LMWH is only partially neutralized by protamine sulfate. **The major advantages of LMWH in comparison to UFH are therefore 1) more predictable pharmacokinetics, which eliminates the need for routine monitoring; 2) subcutaneous administration, which facilitates outpatient administration; and 3) a lower risk of complications including HIT and osteoporosis.**

c) Fondaparinux-

Fondaparinux is a synthetic analog of the specific heparin pentasaccharide sequence that binds to antithrombin with high affinity, triggering conformational activation of the inhibitor. It can be thought of as an “ultra-low molecular weight” heparin. Fondaparinux acts primarily to accelerate antithrombin inhibition of factor Xa, with no significant activity versus thrombin. It has a longer half-life (17-21 hours) than LMWH and is administered subcutaneously once daily. Similar to LMWH, it has predictable pharmacokinetics, does not require coagulation monitoring, and is cleared almost exclusively by the kidneys. Thus, renal insufficiency is a contraindication for use. Fondaparinux does not appear to be associated with heparin-induced thrombocytopenia. The major complication associated with this drug (as is the case with LMWH) is a dose-dependent risk of bleeding. Fondaparinux is not neutralized by protamine sulfate; there is no specific antidote for it. It rarely if ever causes HIT.

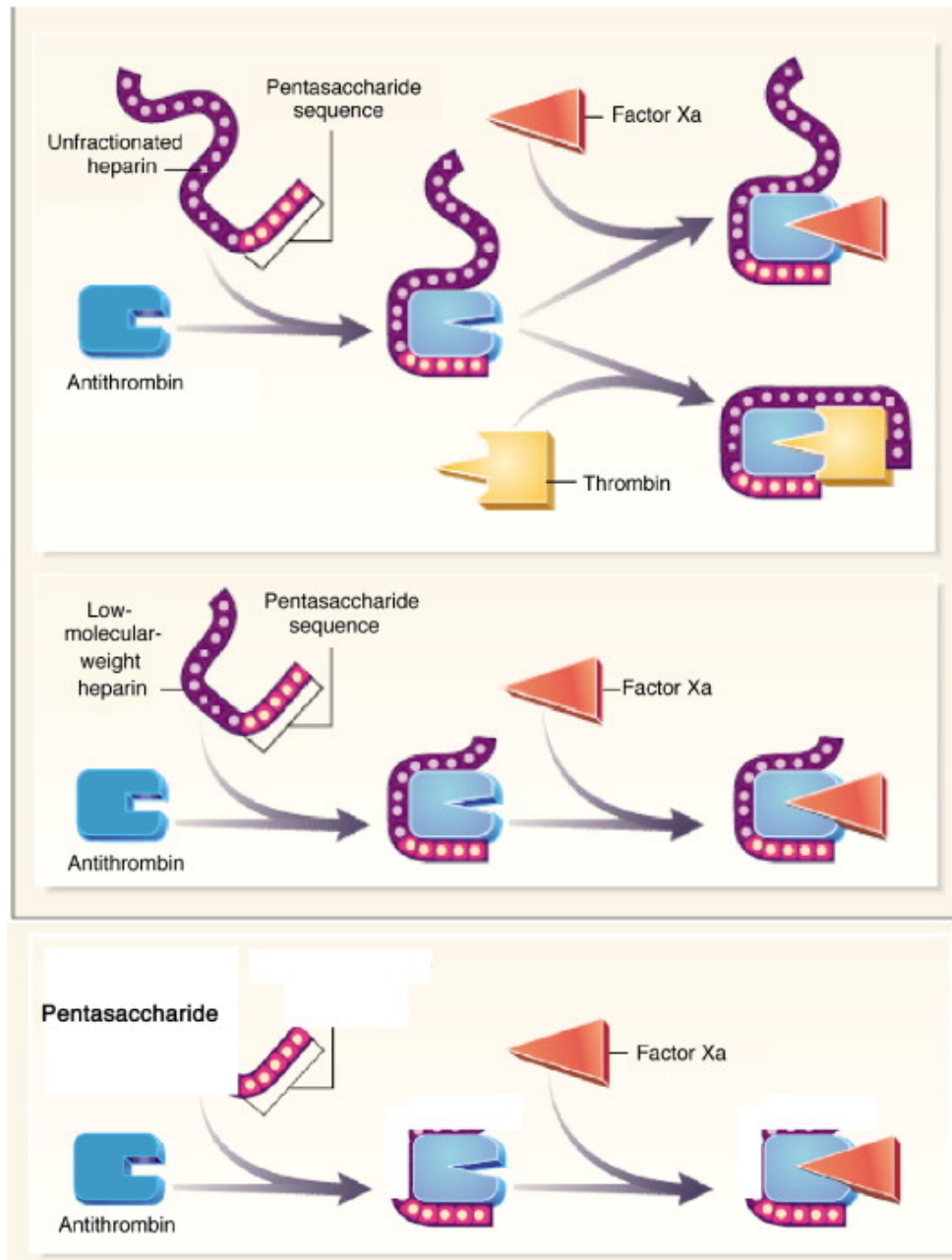


Figure 11.15- Mechanism for heparin-catalyzed inhibition of coagulation proteases. A specific pentasaccharide within the heparin chain binds with high affinity to antithrombin (AT), altering its conformation. Conformational activation of AT markedly accelerates the rate of factor Xa inhibition, but minimally affects thrombin inhibition. Thus, the short chains of LMWH (middle panel) or the isolated pentasaccharide (Fondaparinux) (bottom) are sufficient to accelerate factor Xa inhibition. In contrast, the longer UFH chains simultaneously bind AT and protease, which results in marked acceleration of thrombin inhibition. Thus, UFH accelerates factor Xa and thrombin inhibition via different mechanisms. Note that heparin is not consumed but is released upon formation of the AT-protease complex to catalyze additional rounds of inhibition (Illustration from Middeldorp S. *Thromb Res* 2008; 6:753-62).

4. Indications

- **LMWH/heparin is the drug of choice for initial treatment of deep-vein thrombosis and pulmonary embolism.** Initial treatment with LMWH/heparin is followed by warfarin to decrease risk of recurrence. Because of its favorable pharmacokinetic and side effect profiles, LMWH is now used more often than UFH for this purpose.
- LMWH/heparin is used prophylactically to prevent postoperative venous thrombosis and during acute MI.
- UFH is used in dialysis machines, cardiopulmonary bypass machines and other devices to prevent thrombosis.
- LMWH/heparin does not cross the placenta. **LMWH is the anticoagulant of choice to treat or prevent venous thromboembolism in pregnant women.**

5. Method of administration

- Bolus I.V. injection, continuous infusion, or subcutaneous injection. SC injection allows outpatient administration.
- Heparin and LMWH are large, negatively charged molecules that do not readily cross membranes and are destroyed in the GI tract; therefore, they are not administered orally. LMWH is usually given subcutaneously.

6. Pharmacokinetics

The unpredictability of UFH pharmacokinetics relative to LMWH and Fondaparinux relates to two major factors:

- UFH binds to plasma proteins such as platelet factor 4, endothelial cells, and macrophages, decreasing its bioavailability and leading to a variable dose-response relationship that requires routine monitoring of its anticoagulant effect. In contrast, LMWH does not bind to other blood components as readily as UFH, resulting in more predictable pharmacokinetics that allow weight-based dosing without routine monitoring. Fondaparinux is similar to LMWH in this respect, binding almost exclusively to antithrombin in the blood, with an elimination half-life of 17-21 hours.
- UFH has multiple mechanisms for clearance, with ~50% metabolized in the liver and by endothelial cells and ~50% excreted unchanged by the kidney. Elimination of the longer heparin chains that are predominantly metabolized by the liver results in a longer half-life, and much greater dependence on renal clearance, for both LMWH and Fondaparinux. Thus, LMWH and Fondaparinux have more predictable pharmacokinetics than UFH, but may accumulate in the presence of renal insufficiency. On the other hand, rapid metabolism by the liver makes the use of intravenous UFH advantageous if the patient is in the hospital and expected to require invasive procedures or surgery.

7. Adverse effects of both UFH and LMWH:

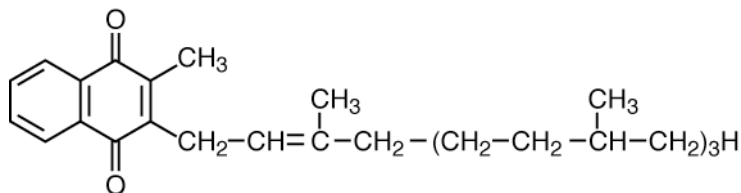
- The most common side effect of excessive doses of heparin (UFH, LMWH or Fondaparinux) is bleeding.
- Mild bleeding can usually be controlled by stopping the drug. Protamine sulfate (positively charged polymer containing multiple arginines) binds to UFH and can reverse the anticoagulant effect. In practice, protamine is not often used as overdose may independently result in a coagulopathy. Protamine only partially neutralizes LMWH since it binds chains >18 saccharides, and has no significant effect on Fondaparinux.
- Heparin-induced thrombocytopenia (HIT, which is often associated with paradoxical thrombosis) occurs in 1-3% of patients treated with UFH. It is significantly less common in patients treated with LMWH. This is discussed in more detail in Chapter 13.
- Hypersensitivity reactions: fever, urticaria, anaphylaxis.
- Alopecia and osteoporosis.

B) Vitamin K antagonists

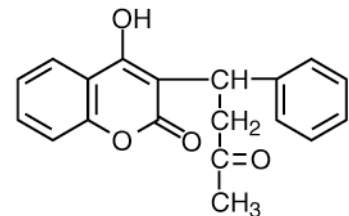
1. Warfarin (Coumadin®)- remains the primary oral drug for chronic anticoagulation.

However, the recent introduction of direct thrombin and factor Xa inhibitors (see below) has begun to impact chronic oral anticoagulant treatment.

a) **Structure.** Similar to vitamin K.



Phylloquinone (Vitamin K₁)



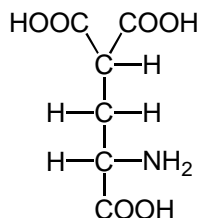
Warfarin

b) Mechanism of action

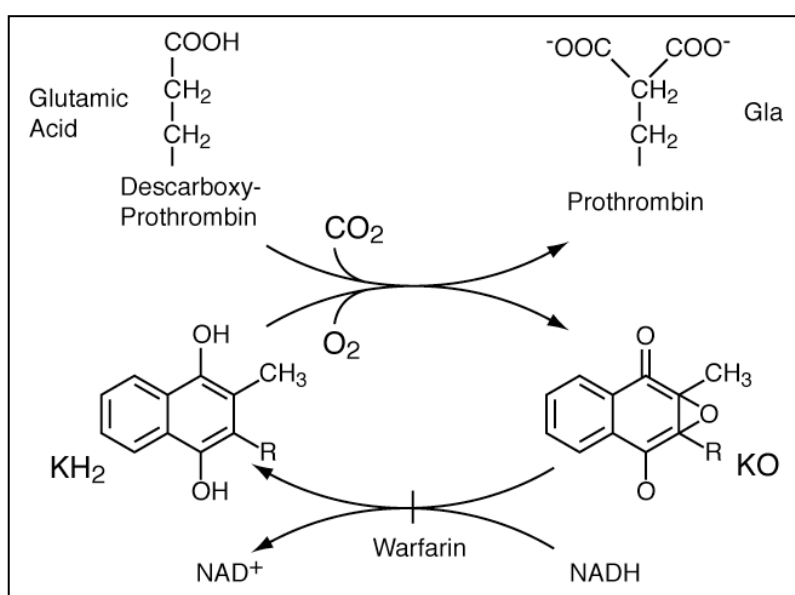
Warfarin acts by interfering with the vitamin K-dependent γ -carboxylation of glutamines within the amino-terminus “Gla domain” of the coagulation proteases. Vitamin K is necessary for this reaction that converts specific glutamyl residues in factors II (prothrombin), VII, IX, and X to “gla” (or γ -carboxyl glutamic acid) residues. The gla residues form tight Ca^{2+} binding sites that are necessary to order the overall structure of the amino-terminus domain, and facilitate the binding of these coagulation factors to the membrane surface. Vitamin K is oxidized during this process and must be “recycled” by reduction, as shown below. Warfarin interferes with reduction of vitamin K and thereby depletes the body of this vitamin. In the absence of vitamin K, under-carboxylated clotting factors with markedly reduced or absent coagulant activity are produced.

(See Chapter 11, part 1.) Although warfarin also affects production of the vitamin K-dependent anticoagulant proteins C and S, its net effect is to retard the clotting process.

“GLA” or γ -carboxyglutamic Acid



Mechanism of warfarin inhibition



c) Pharmacokinetics/Pharmacodynamics/Monitoring:

Warfarin is well absorbed from the gut and is detected in the plasma within one hour. However, the therapeutic effect of this drug does not occur until clotting factor levels decline to about 20-30% of normal. Levels of the vitamin K-dependent zymogens VII, IX, X, and prothrombin gradually decline during warfarin therapy in accordance with their respective plasma half-lives (e.g., factor VII 4-7 hrs, prothrombin 60-72 hrs). The prothrombin time (PT) generally prolongs within 2 days but the full therapeutic effect of the drug is not achieved for several days, when all factors reach steady state. The extent of PT prolongation at a given dose of warfarin varies with different thromboplastin reagents (thromboplastin = mixture of procoagulant lipids and tissue factor). This variation is standardized against a reference thromboplastin, and the result is expressed as the International Normalized Ratio (INR), which is derived from the PT. Use of the INR facilitates comparison of PT values between different clinical laboratories. For most indications, the dose of warfarin is adjusted to achieve an INR of 2.0-3.0. Approximately 5% of patients treated with warfarin develop significant bleeding, thus the relative risk of thrombosis versus hemorrhage must be weighed.

Warfarin has a very complex pharmacology. Its anticoagulant effect is affected by genetic factors, by changes in diet (particularly foods containing vitamin K), and by changes in drug metabolism and serum protein binding induced by other drugs. For these reasons, warfarin's dose-response relationship varies widely among different individuals, and may vary significantly over time in an individual patient due to changes in diet or medication use. **Thus, the INR needs to be carefully monitored in any patient taking warfarin.**

d) Indications

Warfarin is given to prevent recurrence of venous thromboembolism (VTE). It is usually given for 3-6 months following an episode of VTE, but is sometimes prescribed long-term for patients at high risk of recurrence. Physicians also prescribe warfarin to reduce the risk of stroke and other arterial emboli from the heart in patients with **chronic atrial fibrillation, prosthetic heart valves** and other cardiac disorders.

When used to treat patients with acute thrombosis, warfarin is generally overlapped with heparin/LMWH for a minimum of 4 days, and until the INR is between 2.0 and 3.0 for at least 1-2 days.

e) Major side effects

Bleeding. An overdose of warfarin can be counteracted by administering Vitamin K₁ (phytonadione). In cases of severe overdose or major bleeding, rapid correction of warfarin-induced clotting factor deficiency can be accomplished by giving the patient plasma or prothrombin complex concentrate, a plasma-derived preparation enriched in vitamin K-dependent clotting factors. Adequate reversal is determined by monitoring the PT/INR.

Warfarin readily crosses the placenta, and can cause fetal hemorrhage, fetal malformations (first trimester), or even intrauterine death. Therefore **it should never be given to a pregnant woman.** Oral anticoagulants are not secreted in breast milk and so can be given to mothers who are breast-feeding.

Warfarin-induced skin necrosis, characterized by thrombosis of the microvasculature causing lesions that can become necrotic. This rare complication is thought to be due to suppression of protein C production by warfarin.

Drug interactions. Warfarin is particularly susceptible to drug-drug interactions, a feature that constitutes an important disadvantage to its use and that must always be kept in mind when prescribing new medications to warfarin-treated patients.

Several drugs **potentiate** the anticoagulant effect of warfarin.

- **Inhibiting warfarin metabolism.** Many drugs, including phenylbutazone (an NSAID), diphenylhydantoin (phenytoin), (both of which compete for a hydroxylase that metabolizes warfarin), metronidazole, chloramphenicol, cimetidine, cotrimoxazole, disulfiram, and acute alcohol ingestion.
- **Displacement of warfarin from albumin:** aspirin, clofibrate, phenylbutazone, chloral hydrate, several others.

- **Inhibition of platelet aggregation:** aspirin, clopidogrel, others.
- **Decreased Vitamin K synthesis:** antibiotics (much of the Vitamin K in the diet is supplied by bacteria in the GI tract, which can be adversely affected by many antibiotics).

Drugs that **decrease** the anticoagulant effects of warfarin.

- **Stimulation of warfarin metabolism:** barbiturates, glutethimide, chloral hydrate, rifampin, chronic alcohol ingestion

C) Small Molecule Protease Inhibitors

1. Direct Thrombin Inhibitors- small peptides or peptide-mimetics (chemicals that have peptide-like structure) that directly bind to and inhibit the active site of thrombin. These molecules can bind to and inhibit both clot-bound thrombin and circulating thrombin, which may potentially increase their efficacy compared to the antithrombin-heparin complex.

a) Argatroban (intravenous)

- synthetic direct thrombin inhibitor derived from L-arginine (M.W. 527)
- primarily *hepatic clearance*, elimination half-life- 30-51 min
- Monitored by prolongation of the aPTT (target 1.5-3 times control)
- FDA-approved for treatment of heparin-induced thrombocytopenia

b) Bivalirudin (intravenous)

- Bivalirudin is a 20 amino acid peptide (MW 2,180) that binds to both substrate (fibrinogen)-binding site and active site of thrombin.
- primarily *renal clearance*, elimination half-life- 25 min
- monitored by activated clotting time (bedside test similar to aPTT done in procedure room)
- FDA-approved for use in percutaneous coronary angioplasty (usually given along with antiplatelet agents) and heparin-induced thrombocytopenia

c) Dabigatran (oral)

- Dabigatran (FDA approved in 2010) was the first oral anticoagulant alternative to warfarin, which had been in clinical use for over 50 years.
- Dabigatran is administered as the prodrug, dabigatran etexilate mesylate, which is activated by esterases in the blood and liver.
- Small molecule reversible inhibitor of thrombin, (M.W. 724) with a structure similar to benzamidine-based protease inhibitors. Addition of a hydrophobic side chain created the orally-absorbed prodrug.
- Only about 6% of orally administered drug is actually absorbed.
- Twice daily oral dosing, peak drug levels 1.5-3 hours after dose.
- Primarily renal clearance, elimination half-life 12-17 hours
- Dose-response relationship is more predictable than for warfarin; no routine monitoring.
- No proven antidote; can be removed by dialysis.

2. Direct Factor Xa inhibitors

Rivaroxaban (oral)

- small molecule (MW 436) that reversibly inhibits factor Xa active site
- Once or twice daily oral dosing, peak effect 2-4 hours after dose
- Clearance by liver, biliary excretion, and kidneys; elimination half-life 5-9 hours
- FDA-approved for prevention of venous thromboembolism after knee or hip surgery (once daily dosing), for stroke prevention in atrial fibrillation, and for treatment and secondary prevention of VTE.

FYI

A second oral direct thrombin inhibitor, apixaban, has recently been approved by the FDA. A third drug, edoxaban, is currently in clinical trials.

3. Advantages and disadvantages of new oral anticoagulants vs warfarin:

- Initial cost of the new agents is ~10-fold greater than warfarin. A portion of this cost differential is recouped because these drugs have predictable pharmacokinetics, fewer drug interactions, and do not require routine monitoring.
- Onset of therapeutic action within a few hours compared to 4-5 days for warfarin.
- More predictable dose-response relationship than warfarin allows administration without routine monitoring of anticoagulant effect.
- More difficult to monitor, so you cannot reliably assess compliance or overdose.
- Impaired renal or hepatic function may lead to accumulation of drug. The PT/INR and aPTT are not sufficiently sensitive to the drugs' effects to reliably detect this problem.
- No antidote/reversal agent.
- Several large randomized trials in patients with chronic atrial fibrillation demonstrated that the new oral agents had similar or somewhat better efficacy and safety profiles than warfarin, and in particular were associated with lower risks of hemorrhagic stroke.
- Trials of the new agents for the prevention and treatment of venous thromboembolism have generally shown similar efficacy and safety to warfarin.

III. Antiplatelet Therapy

A. Strategies for inhibition of platelet function

Arterial thrombi form under high-flow conditions and are platelet dependent. Because platelets dominate arterial thrombi (compared to venous thrombi), strategies for prevention and treatment of clinical events triggered by arterial thrombosis (including MI, stroke, limb ischemia) usually target platelets. Antiplatelet drugs are often used together with heparin-based therapies in the acute setting, particularly in coronary disease.

Therapies are aimed at key steps in platelet activation and aggregation:

- Signaling events triggered by binding to collagen exposed in the injured vessel wall, ADP, thrombin, or fibrinogen lead to the release of arachidonic acid (AA) and activation of the cyclooxygenase pathway by which AA is converted to TXA₂.
- A key step in platelet activation is the resultant “activation” of platelet glycoprotein IIb-IIIa, the fibrinogen receptor. Activated platelets form aggregates by binding to soluble fibrinogen in plasma via GPIIb-IIIa.

B. Inhibitors of Platelet Signaling

1. Aspirin

a. Structure. Nonsteroidal anti-inflammatory agent (acetylsalicylic acid).

b. Mechanism of action (see Figure 11.16). Inhibits platelet activation by irreversibly inhibiting cyclooxygenase. In platelets, this enzyme converts arachidonic acid to thromboxane A₂, a powerful platelet activator. The inhibition of cyclooxygenase is irreversible, resulting in a suppression of platelet aggregation for the life of the platelet - 7-10 days.

Note that higher doses of aspirin also inhibit cyclooxygenase in the vascular endothelium, resulting in decreased prostacyclin production. This would oppose the antithrombotic effect of aspirin. Because endothelial cells, unlike platelets, can make more cyclooxygenase, the antiplatelet effect of aspirin predominates.

Note: Other NSAIDs also inhibit COX I but do so reversibly. Because these drugs compete with aspirin for binding to COX1, they can interfere with the anti-platelet effects of aspirin.

c. Indications

- **Unstable angina.** The incidence of subsequent MI or ischemic cardiac death is decreased in patients treated with aspirin.
- **Prevent thrombosis following coronary artery bypass.** 20% of grafts occlude by thrombosis within the first year.
- **Coronary angioplasty.** This procedure physically disrupts the occluding atherosclerotic plaque or thrombus with a catheter inserted into the artery. There is significant risk of reocclusion since the procedure damages the endothelial lining of the vessel wall, providing a site for thrombus formation. Trials with the combination of dipyridamole and aspirin suggest a reduction in the incidence of reocclusion by decreasing thrombus formation.
- Prevent thrombosis of arterial venous shunts in patients undergoing long-term hemodialysis.
- Prevention of thrombosis and stroke for patients with prosthetic heart valves. Aspirin is significantly less effective than warfarin in this regard.
- **Prevention of acute myocardial infarction (MI).** Prophylactic aspirin reduces the incidence of fatal and non-fatal MI by almost 50% for men and women with no previous ischemic heart disease, and significantly decreases subsequent MI and death in patients with a history of prior MI.

- **Transient ischemic attack (TIA).** This is a syndrome of the cerebral circulation that may be initiated by atherosclerotic lesions. 25-40% of patients with TIA eventually have cerebral infarction (stroke). Prophylactic aspirin has been shown to reduce the chances of another stroke.

d. Method of administration. Oral. Low doses (50-150 mg/day) inhibit the platelet cyclooxygenase more than the vascular enzyme, resulting in net platelet inhibition.

e. Major side effects. Prolonged bleeding time, causing potential increased incidence of hemorrhagic stroke and GI bleeding, especially at higher doses. Other side effects include rash, bruising, tinnitus, and gastritis.

f. Drug interactions. If used with other anticoagulants, aspirin often potentiates antiplatelet effects. It displaces bound warfarin from albumin. Avoid using with other GI irritants and ototoxic drugs.

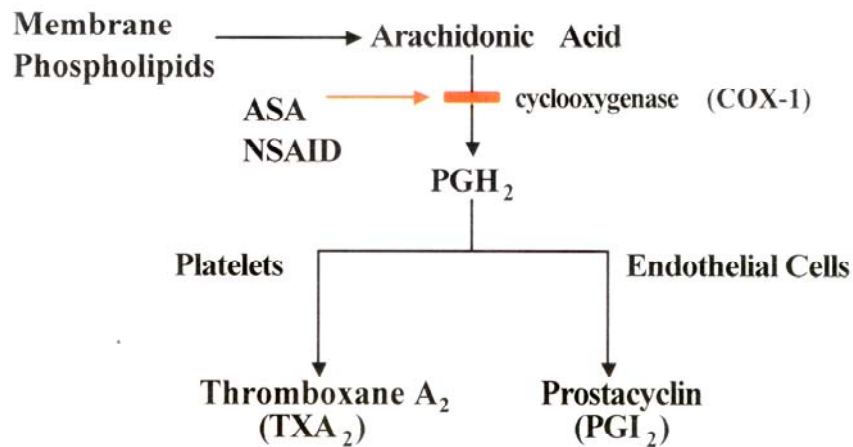


Figure 11.16 Mechanism of ASA action. Arachidonic acid is converted by cyclooxygenase (COX-1) via intermediates primarily to thromboxane A₂ in platelets (a platelet agonist and vasoconstrictor), or prostacyclin in endothelial cells (a platelet antagonist and vasodilator). Because the anucleate platelet has very limited protein synthetic abilities, ASA essentially blocks thromboxane A₂ production for the lifetime of the platelet by acetylating the COX-1 active site (irreversible inhibition). In contrast, endothelial cells synthesize additional COX-1 to overcome effects on prostacyclin production. Other NSAIDs (ibuprofen, etc.) act similarly but in contrast to ASA, reversibly inhibit the COX-1 active site.

- 2) **The other side of the coin: Selective COX-2 inhibitors and the risk of cardiovascular events.** The COX enzymes have similar structures, but markedly different expression patterns. In contrast to the constitutive expression of COX-1 in platelets, COX-2 is rapidly inducible in the endothelium (and other tissues) by inflammatory stimuli. Selective inhibition of COX-2 inhibits production of prostacyclin (PGI₂) and related compounds by the endothelium, without inhibition of thromboxane A₂ generation by COX-1 in platelets. The use of these drugs has been associated with increased risk of cardiovascular events, leading to the recent withdrawal of rofecoxib (Vioxx) from the market. This effect is likely class-

specific, and selective COX-2 inhibitors should be avoided in patients with known cardiovascular risk factors.

2. ADP receptor inhibitors: Clopidogrel and related drugs inhibit ADP-dependent platelet aggregation, probably via covalent modification of the platelet ADP receptor.

a. Clopidogrel selectively and irreversibly blocks ADP binding to platelets. Its effects are due to an active liver metabolite.

- **Structure.** A thienopyridine.
- **Mechanism of action.** Clopidogrel is converted by the liver (cytochrome P450) to an active metabolite that interferes with ADP-induced platelet aggregation by binding to the platelet ADP receptor.
- **Indications.**
 - TIA (recent studies suggest it is slightly superior to aspirin in reducing the risk of stroke and death), completed thrombotic stroke.
 - Acute coronary syndrome (often used together with aspirin)
 - Prevention of coronary stent occlusion.
- **Method of administration.** Oral.
- **Pharmacokinetics:** 20-30% of individuals have a polymorphism in the CYP2C enzyme that results in lower production of the active metabolite, and a significantly reduced antiplatelet effect.
- **Major side effects**
 - Immunologically mediated thrombocytopenia, neutropenia.
 - Hemorrhage (GI and intracranial)
 - Clopidogrel can inhibit cytochrome P-450, and thereby may block the metabolism of other drugs, including phenytoin, tolbutamide, warfarin, fluvastatin, and tamoxifen.

b. Prasugrel

- **Structure.** A thienopyridine.
- **Mechanism of action.** Interferes with ADP-induced platelet aggregation by binding to the platelet ADP receptor.
- **Indications.**
 - Prasugrel is currently only approved in US for use during percutaneous coronary intervention (PCI). Its effectiveness exceeded clopidogrel during PCI.
- **Method of administration.** Oral.
- **Pharmacokinetics:** Prasugrel is metabolized more efficiently than clopidogrel and thus has a more predictable dose-response relationship.

- **Major side effects**

- Side effects are similar to clopidogrel, however there is a higher risk of bleeding than with clopidogrel.
- Prasugrel is contraindicated in patients with a history of stroke or TIA, due to risk of hemorrhagic transformation.

c. Dipyridamole (originally classified as a vasodilator)

- **Structure.** Pyrimidine analog.
- **Mechanism of action.** Dipyridamole increases cyclic AMP levels in platelets due to a weak inhibition of phosphodiesterase activity, thereby decreasing platelet reactivity.
- **Indications.**
 - In patients with artificial heart valves, use in combination with anticoagulants to decrease thromboembolism.
 - Dipyridamole is often given in conjunction with aspirin - i.e., **Aggrenox**, a combination of 25 mg aspirin and 200 mg extended-release dipyridamole. Aggrenox appears to be more effective than aspirin alone in preventing stroke in patients with prior TIA.
- **Method of administration.** Oral.
- **Major side effects.** Headache, dizziness, flushing, nausea, diarrhea.

C. Glycoprotein (GP) IIB-IIIa Antagonists

Note that these agents will inhibit platelet aggregation induced by any platelet agonist because they inhibit the final common mechanism of platelet aggregation— fibrinogen binding to GP IIB-IIIa. *antibody or peptidomimetic block of platelet aggregation via this platelet surface receptor.*

1. Abciximab

a. Structure. Humanized chimeric Fab version of a mouse monoclonal antibody against the platelet glycoprotein IIB-IIIa fibrinogen receptor.

b. Mechanism of action. Abciximab binds to glycoprotein IIB-IIIa on platelets and inhibits binding of fibrinogen and vWF, thereby inhibiting platelet aggregation.

c. Indications.

- Adjunct therapy to percutaneous coronary intervention (PCI), i.e., patients undergoing angioplasty, atherectomy, or stent placement.
- Also used in cases of unstable angina, where patient does not respond to conventional therapy, non-Q-wave myocardial infarction.

d. Method of administration. I.V. (since it is a protein, it is degraded if administered orally).

e. Major side effects. Bleeding, thrombocytopenia (less than 1%).

2. Eptifibatide, Tirofiban

a. Structure. These drugs mimic the RGD (arginine-glycine-aspartate) peptide sequence, which is the ligand for GPIIb-IIIa. Eptifibatide is a cyclic peptide containing six amino acids, derived from a rattlesnake venom peptide. Tirofiban is a peptidomimetic.

b. Mechanism of action. These drugs bind to glycoprotein IIb-IIIa on platelets and inhibit fibrinogen binding, thereby inhibiting platelet aggregation.

c. Indications. Percutaneous coronary intervention, unstable angina, non-Q-wave MI.

d. Method of administration. I.V.

Oral versions were tried, but had partial agonist function, and resulted in immune response against drug.

e. Major side effects. Bleeding.

IV. Thrombolytic Therapy

Thrombolytic therapy is sometimes used to treat patients with established thrombosis, in order to dissolve the clot and limit or reverse tissue and organ injury caused by vessel occlusion.

Thrombolytic drugs convert the inactive zymogen plasminogen to the active enzyme plasmin. Plasmin breaks down fibrin (fibrinolysis) and leads to clot dissolution. The major risk of thrombolytic therapy is bleeding.

Clots become more resistant to lysis as they age, and tissue injury caused by vessel occlusion tends to become irreversible over time, so thrombolytic therapy has the greatest benefit when it is initiated early after clot formation. Clot lysis may expose clot-bound thrombin, and thus enhance platelet activation and aggregability. Therefore thrombolytic drugs are often given in conjunction with antiplatelet or antithrombotic drugs, such as aspirin and heparin.

1. Agents-

- Tissue plasminogen activator (tPA) and modified recombinant forms (reteplase and alteplase)

2. Mechanism of action- These agents convert plasminogen to plasmin, which then digests the fibrin thrombus. Because these drugs activate clot-bound and not circulating plasminogen, most of the fibrinolytic activity is confined to the clot. However, a systemic fibrinolytic state with circulating plasmin is sometimes observed during therapy, resulting in decreased fibrinogen, factor V, and factor VIII levels as well. Broader use of fibrinolytic agents is primarily limited by the ~0.5 to 1% incidence of cerebral hemorrhage, which is often fatal, noted in large myocardial infarction trials.

3. Indications for fibrinolysis:

- Acute myocardial infarction. When given within 2-3 hours of symptom onset thrombolytic drugs can limit or reverse ischemic damage, improve cardiac function, and decrease mortality.

- Acute peripheral arterial obstruction (limb salvage)
- Ischemic stroke. When given within 3 hours of symptom onset), thrombolytic therapy improves neurologic outcome and decreases mortality in acute ischemic stroke. The major risk is conversion of an ischemic to a hemorrhagic stroke with potentially fatal intracerebral bleeding
- Massive pulmonary embolism with hemodynamic compromise
- Massive proximal (thigh or above) DVT with limb-threatening venous hypertension.
- Thrombolytic therapy is used far more often to treat arterial than venous occlusion, because of the greater potential for irreversible tissue injury after arterial occlusion. Its use in venous thromboembolic disease has not conclusively been shown to improve long-term clinical outcomes.

4. Mode of administration

Thrombolytic agents are usually administered I.V., but are sometimes administered directly to the clot site by an intravascular catheter.

5. Thrombolytic drugs in clinical use

A. Tissue Plasminogen Activator (tPA): Alteplase, Reteplase

- **Structure.** This plasminogen activator is a naturally occurring serine protease present in most normal and neoplastic cells. $M_r = 68,000$ Daltons. The therapeutic agent is manufactured by recombinant DNA technology (rTPA). Alteplase is unmodified and Reteplase has several deleted amino acids.
- **Mechanism of action.** tPA converts plasminogen to plasmin, which lyses fibrin clots. The thrombolytic effects of rTPA are specific for fibrin-bound plasminogen in the thrombus, with much less effect on circulating plasminogen. The systemic lytic effect can still be significant and cause bleeding.
- **Method of administration.** rTPA has a very short half-life in plasma and is usually given by continuous IV infusion.
- **Major side effects.** Bleeding.

6. Factors Determining the Success of Thrombolytic Therapy

- **Time of administration of thrombolytic agent** - the earlier, the better. New thrombi are more readily lysed, and less ischemic damage has occurred.
- **The characteristic of the thrombus** - a thrombus that completely occludes a vessel or is large will have interior regions that are inaccessible to the thrombolytic therapy.
- **Reocclusion** - occurs when the thrombotic stimulus is still present, such as a severe atherosclerotic lesion. Exposure of clot-bound thrombin may also contribute to this problem. To prevent reocclusion, prolonged periods of thrombolytic treatment or combination therapy with heparin or aspirin has been used.

V. Hemostatic Therapy

Hemostatic Drugs:

Desmopressin
Aminocaproic Acid/Tranexamic acid
Factor VIIa
Vitamin K

1. Desmopressin (DDAVP)

Desmopressin is a synthetic peptide replacement for vasopressin. It is a stereoisomer that has less effect on blood pressure than vasopressin. Desmopressin promotes the release of vWF and factor VIII stored in endothelial cells.

1. Indications:

- Type I vWF disease
- Mild hemophilia due to factor VIII deficiency
- Thrombocytopenia

2. Pharmacokinetics: Orally bioavailable. Half-life = 1-3 hours. Renal clearance.

3. Side effects:

- Increases water resorption from the kidneys (also prescribed for bedwetting)
- Hyponatremia
- Headaches, nausea, seizures are possible

2. Aminocaproic Acid (also known as Amicar, ϵ -aminocaproic acid, or 6-aminohexanoic acid) Aminocaproic acid is an anti-fibrinolytic. A lysine analog, it binds to and inhibits plasminogen activation. **Tranexamic acid** (aminomethyl-cyclohexane carboxylic acid) has similar properties.

1. Indications:

- Treatment of excessive post-operative bleeding
- Reduce blood loss during coronary bypass
- Treatment of bleeding associated with administration of thrombolytic drugs
- Prevention of bleeding in patients with severe, chronic thrombocytopenia
- A recent clinical trial showed that administration of tranexamic acid to trauma patients within 3 hours of injury decreased the likelihood of death from bleeding

2. Pharmacokinetics:

Administered orally or IV

Renally cleared

Half life = 2 hrs.

3. Side effects:

- GI distress: nausea, vomiting, abdominal pain, diarrhea.
- Increased thrombosis.

3. Factor VIIa

Recombinant human factor VIIa (rhFVIIa) is administered IV.

Indications:

- Hemophilia patients who have developed inhibitors or antibodies against replacement coagulation factors; treatment of patients with acquired factor VIII inhibitors
- Uncontrollable hemorrhage, intracerebral hemorrhage

4. Vitamin K

Several of the coagulation factors are vitamin K-dependent for their production.

1. Indications:

- Acute elevation of INR, such as during warfarin therapy
- Patients who require surgery (and who have an elevated INR)
- Patients with serious bleeding

2. Administration:

- Oral (vitamin K concentrate)
- IV (not preferred, as allergic reactions can occur)
- High doses (over 10 mg) are often used, but overcorrection and warfarin resistance can occur. Low doses (0.5-2.5 mg) reverse anticoagulation within 24 hours with less risk of overcorrection.

CHAPTER 12

BLEEDING DISORDERS

Key Concepts:

- Interpretation of screening tests for bleeding disorders
- Pathogenesis and diagnosis of:
 - Immune thrombocytopenic purpura (ITP)
 - von Willebrand disease
 - Hemophilia A and B
 - Vitamin K deficiency
 - Disseminated intravascular coagulation (DIC)
 - Thrombotic thrombocytopenic purpura (TTP)

Learning Objectives:

1. Describe the elements of the history and physical exam that are important for clinical evaluation of bleeding disorders.
2. Describe the relationship between platelet count and bleeding risk in conditions associated with decreased production or increased destruction of platelets.
3. List at least four conditions that may cause failure of platelet production.
4. Describe and compare the pathophysiology and treatment of the childhood (acute) and adult (chronic) forms of immune thrombocytopenic purpura (ITP), and drug-induced immune thrombocytopenia.
5. Describe the pathophysiology, genetics, and the clinical and laboratory characteristics of the most common form of von Willebrand disease (type I vWD).
6. Describe the pathophysiology, genetics, and the clinical and laboratory characteristics of hemophilia A and B.
7. Describe the pathophysiology of Vitamin K deficiency, and list several causes of this condition.
8. Be able to explain how to use laboratory tests to distinguish coagulation factor deficiency from an inhibitor of coagulation.
9. List several important causes of disseminated intravascular coagulation (DIC), and describe the pathophysiology and major clinical and laboratory characteristics of this syndrome.
10. Describe the different laboratory findings associated with the presence of liver disease, DIC, and warfarin treatment or vitamin K deficiency.
11. Describe the distinguishing laboratory characteristics of microangiopathic hemolytic anemia, and the pathophysiology and major clinical features of thrombotic thrombocytopenic purpura (TTP), including the roles of von Willebrand factor and ADAMTS-13.

I. Approach to the Patient with a Possible Bleeding Disorder

Although the discussion of bleeding disorders frequently emphasizes laboratory testing, the patient history is often the most informative tool in the evaluation of these disorders. The history can provide clues as to whether a clinically significant bleeding diathesis is present, what type of defect is likely, and whether this represents a hereditary or acquired condition. Examination of the skin, oropharynx, abdomen, and joints may suggest the type of defect and severity of the suspected disorder. Thus, a careful history and physical exam will often focus the subsequent laboratory evaluation and choice of therapy.

A. Important Elements of the Patient History

- 1. Is the patient bleeding from single or multiple sites?** The former should prompt an evaluation of local anatomy and pathology. The latter is suggestive of a systemic defect in hemostasis.
- 2. What is the pattern of bleeding?** Mucocutaneous versus deep tissue/joint bleeding? In general, bleeding limited to the skin and mucosal surfaces suggests thrombocytopenia or a defect in platelet or von Willebrand factor function (primary hemostasis). Development of oral mucosal “blood blisters” is usually an indication of clinically severe thrombocytopenia. Deep tissue bleeding, on the other hand, suggests a defect in the soluble coagulation factor response (secondary hemostasis). Retroperitoneal bleeding or hemarthrosis are particularly associated with defects in secondary hemostasis.
- 3. Evaluate all potential sites of bleeding.** Inquire about excessive or spontaneous bruising, nosebleeds (epistaxis) and gum bleeding, hematemesis, blood in the urine (hematuria) or stool (hematochezia, melena), and unusually heavy or prolonged menstrual flow (menorrhagia).
- 4. Has the patient had previous hemostatic challenges,** including surgery, major trauma, or tooth extractions? If so, was there excessive bleeding? Some bleeding tendencies, such as mild hemophilia and von Willebrand disease (vWD), may not be apparent in the absence of a challenge.
- 5. Does the patient have a history of chronic anemia?** Have they previously received blood products or been treated with chronic iron replacement?
- 6. Does the patient have a family history of bleeding problems?** Are both male and female family members affected?
- 7. Has the patient taken any medications in the past 1-2 wks?** Especially note aspirin, NSAIDs, other antiplatelet agents (clopidogrel, glycoprotein IIb/IIIa inhibitors), cold remedies (often contain aspirin), alcohol use, and herbal remedies.
- 8. Does the patient have any underlying medical conditions?** Of particular relevance are liver disease and uremia.

B. Physical Exam

1. **Skin-** Petechiae (pinpoint bleeding in skin), generalized purpura or ecchymoses (larger bruising), perifollicular purpura (scurvy), striae, and telangiectasias. Stigmata of liver disease include spider angiomas and palmar erythema. Petechiae may be diffuse, but are especially common in the lower extremities. Very large ecchymoses are often pathologic, especially when they occur spontaneously in the absence of trauma.
2. **Oral mucosa-** Palatal petechiae, buccal mucosal hematomas or “blood blisters”, gum bleeding, and telangiectasias. In particular, “blood blisters” on the buccal mucosa suggest severe thrombocytopenia.
3. **Splenomegaly-** An enlarged spleen will sequester platelets, and may be a sign of underlying liver disease.
4. **Joint deformities-** Chronic arthropathy may indicate moderate or severe hemophilia.

C. Laboratory Evaluation (see Chapter 11 for descriptions of individual tests)

The laboratory evaluation should be guided by the clinical history. Initial screening of platelet function includes a platelet count, review of peripheral smear, and a platelet function screen such as the PFA-100 (see below). Initial evaluation of von Willebrand factor (vWF) function includes a von Willebrand antigen level, factor VIII activity, and ristocetin cofactor activity (or other functional test). Initial screening of coagulation function includes the prothrombin time (PT) and activated partial thromboplastin time (APTT). Evaluation for dysfibrinogenemia, α_2 -antiplasmin deficiency (an indicator of excessive fibrinolytic activity), or factor XIII activity may also be undertaken in selected patients. Our ability to detect coagulation defects in the laboratory is generally quite good. In contrast, our ability to assess fibrinolysis is only fair, and assessment of platelet function is relatively crude due to the complex nature of the platelet response.

III. Inherited Bleeding Disorders

A significant body of knowledge exists regarding the genetics and physiology of human bleeding disorders. **Most inherited bleeding disorders result from single gene defects.** Deficiency states have been described for all of the physiologically relevant coagulation proteins (fibrinogen, prothrombin, factors V, VII, VIII, IX, X, XI, and XIII). **Hemophilia A (factor VIII deficiency), hemophilia B (factor IX deficiency), and von Willebrand disease together account for approximately 80% of inherited clinical bleeding disorders.** These conditions are relatively common because they are either X-linked (in the case of the hemophilias) or dominantly inherited (most forms of von Willebrand disease). Inherited deficiencies of factors V, VII, X, XI, XIII, and prothrombin are recessively inherited (that is, they do not cause bleeding in the heterozygous state) and rare. Likewise, inherited defects in platelet production and function are relatively rare.

A. Hemophilia A and B (factor VIII and factor IX deficiency)

These disorders have very similar clinical phenotypes, which is not surprising since they are components of the same enzyme complex (intrinsic tenase – see Chapter 11). Factor VIII deficiency (Hemophilia A) represents 80-85% of congenital hemophilia. **These disorders both demonstrate X-linked inheritance, and the severity of the phenotype depends on specific factor activity (<1% activity = severe, 1-5% activity = moderate, 5-30% activity = mild). Severe hemophiliacs demonstrate spontaneous bleeds into joints and deep tissue and require factor replacement on an ongoing basis.** They sometimes develop high titer inhibitors (antibodies against transfused clotting factor) that may greatly complicate therapy. Moderate hemophiliacs demonstrate similar bleeds, more often precipitated by minor trauma. Mild hemophiliacs may be asymptomatic until challenged by significant trauma or surgery. Delayed bleeding into deep tissue and joints is typical, and can lead to chronic arthropathy and disability. Until recently, blood-borne HIV and hepatitis virus infection were important causes of morbidity and mortality for hemophiliacs. Fortunately, the clotting factor replacement products in current use are very unlikely to transmit such viral infections. Therapy consists of specific **factor replacement** with concentrated factor VIII or IX (purified from human plasma and subjected to treatments to inactivate viruses, or synthesized using recombinant DNA technology). Gene therapy is an active field of hemophilia research.

FYI

Factor XI deficiency

Factor XI deficiency is a somewhat variable, mild bleeding tendency in which symptoms do not always correlate with the plasma factor XI level. It is relatively prevalent in the Ashkenazi Jew population with a gene frequency of 4.3%, but also occurs in non-Jewish populations. Family members tend to have similar hemorrhagic symptoms. Increased bleeding may occur after aspirin ingestion or surgery. Common symptoms include menorrhagia, epistaxis, post-partum bleeding, and hematuria. When replacement therapy is required, fresh frozen plasma (FFP) is given to achieve factor XI target levels of at least 30-45%, depending on the nature of the procedure.

FYI

Rare Bleeding Disorders

1. Prothrombin, Factor V, VII, or X Deficiencies

These conditions are rare, autosomal recessive disorders in which affected individuals are homozygotes or compound heterozygotes.

2. Factor XIII Deficiency

This rare condition classically presents with umbilical stump bleeding in the neonate. Intracranial hemorrhage is a common cause of death, and the condition is also associated with defective wound healing and spontaneous abortion.

3. Afibrinogenemia/Dysfibrinogenemias

The former is very rare homozygous recessive disorder with variable bleeding tendency. The latter is a more common autosomal dominant disorder that may be associated with bleeding or thrombosis.

B. von Willebrand Disease (vWD)

von Willebrand disease (vWD) is heterogeneous disease in which there is a **quantitative or qualitative deficiency** of von Willebrand factor, usually accompanied by a parallel decrease in factor VIII activity. Consistent with the critical role of vWF in platelet function, these disorders are generally characterized by mucocutaneous bleeding, but soft tissue/joint tissue bleeding may also be observed in variants associated with severely depressed factor VIII levels. The latter finding is due to the fact that vWF acts as a carrier protein that prolongs the plasma half-life of factor VIII five-fold. **The most common form of vWD is inherited in an autosomal dominant fashion and characterized by a quantitative decrease in vWF. Generally, a proportional decrease in vWF antigen and activity and factor VIII activity is observed.** If there is a significant disparity between vWF antigen and activity (antigen higher than activity) this suggests a qualitative or functional defect in vWF (type 2 vWD). Except for rare patients who have complete or near-complete deficiency of vWF, the bleeding tendency in vWD is typically in the mild to moderate range.

FYI

Establishing the diagnosis of type I vWD (the most common form) can be problematic because slightly low levels of vWF and mild bleeding symptoms are common in the general population. The population distribution of vWF antigen levels is broad and affected by a number of genetic factors (prominently blood type) that are distinct from the vWF locus, as well as environmental influences such as hormonal status, infection, pregnancy, etc. Due to these difficulties, it has been suggested that a definitive diagnosis of Type I vWD be reserved for patients that have bleeding symptoms, low vWF antigen (usually <20-30%), and a positive family history. However, moderately low levels of vWF are a risk factor for bleeding, and can be treated as the clinical situation dictates.

Type I vWD is usually treated by administration of DDAVP (desmopressin, a vasopressin analog – see Chapter 11 part 2), which stimulates the release of endogenous vWF from endothelial cells. For forms of the disease in which an abnormal vWF protein is expressed (type 2 or qualitative deficiency), or for severe (type 3) deficiency, replacement therapy with clotting factor concentrates that contain the factor VIII-vWF complex is preferred. Cryoprecipitate also contains large amounts of vWF, but is usually avoided because it has not been treated to inactivate viruses. Antifibrinolytic agents such as ϵ -aminocaproic acid (Amicar™) or tranexamic acid are also useful for mucocutaneous bleeding.

C. Inherited Platelet Disorders

Deficiency of surface glycoprotein complexes

Inherited deficiencies of the major platelet surface glycoproteins cause mild to moderate bleeding. Although rare, these disorders are important “experiments of nature” that provide important insights into the role of these glycoprotein complexes in platelet function. **Congenital deficiency of the Gp IIb/IIIa complex is known as *Glanzmann’s thrombasthenia*.** The hallmark of this disorder is deficient platelet aggregation in response to multiple agonists, as this receptor is critical for the final step in platelet aggregation, the binding of adhesive proteins such as fibrinogen that cross-link platelets.

Congenital deficiency of Gp Ib is known as *Bernard-Soulier syndrome*. This results in platelets with defective vWF-dependent adhesion. Patients with this disorder also usually have mild thrombocytopenia with large circulating platelets.

FYI

Additional inherited platelet function defects
These include a heterogeneous assortment of mutations in secretion and signal transduction pathways, absence of platelet granules, giant platelet syndromes (macrothrombocytopenia), and congenital thrombocytopenias associated with bone marrow/immune defects.

IV. Acquired Bleeding Disorders

A. Quantitative Platelet Defects (Thrombocytopenia)

1. Classification by mechanism

Once the presence of thrombocytopenia (decreased platelet number) has been confirmed by review of the peripheral smear, it is helpful to classify the process as resulting from one of the following mechanisms: decreased bone marrow production, sequestration of platelets in the spleen, or increased peripheral destruction of platelets.

- **Decreased Production-** congenital (rare), primary bone marrow disorders such as leukemia (Chapter 8), aplastic anemia (Chapter 1), and myelodysplastic syndrome (Chapter 8), or secondary bone marrow suppression due to cytotoxic drugs, radiation, viral infection, nutritional deficiencies, marrow replacement by fibrosis, malignancy. In most instances there are also reductions in the white cell and red blood cell lines (pancytopenia). A bone marrow aspirate and biopsy is often needed to evaluate such cases.
- **Sequestration-** The fraction of platelets that are sequestered in the spleen increases with spleen size. Thus, disorders associated with a large spleen may result in mild to moderate thrombocytopenia. This includes conditions that cause primary enlargement of the spleen such as hematologic malignancies (e.g., myeloproliferative disease – see Chapter 8) or infection, and secondary enlargement due to liver disease with portal vein hypertension. Evaluation requires a careful physical exam and/or imaging of the abdomen. Splenic sequestration *per se* rarely causes bleeding, since the sequestered platelets can re-enter the circulation and contribute to hemostasis.
- **Increased Destruction/Utilization-** This category includes the most common etiologies of thrombocytopenia, which include immune and non-immune-mediated mechanisms.
 - Causes of **immune-mediated** thrombocytopenia include **idiopathic thrombocytopenic purpura (ITP)**, drugs, autoimmune disease, and viral infection including HIV.

- Causes of **nonimmune-mediated** thrombocytopenia include sepsis, **disseminated intravascular coagulation (DIC)**, and **thrombotic thrombocytopenic purpura (TTP)**.

2. Bleeding risk and severity of thrombocytopenia

- **Mild** thrombocytopenia (platelet count 60-150,000/ μ l) is generally asymptomatic.
- **Moderate** thrombocytopenia (20-50,000/ μ l) is associated with bleeding in response to surgery or trauma.
- **Severe** thrombocytopenia (<20,000) is associated with spontaneous bleeding symptoms (bruising, petechiae, and mucous membrane bleeding).
- Patients with platelets < 10,000/ μ l have a significant risk of life-threatening hemorrhage and may be candidates for prophylactic platelet transfusions.

The mechanism of thrombocytopenia and the concomitant use of anti-platelet or anticoagulant medications influence the bleeding risk. At any given platelet count, decreased production is associated with higher risk than increased destruction, because in the latter case the platelets are likely to be younger, larger, and more active (due to high turnover). As would be expected, the use of aspirin or other anticoagulant or antiplatelet drugs increases the bleeding risk associated with thrombocytopenia.

3. Idiopathic (autoimmune) thrombocytopenic purpura (ITP) is the most common cause of isolated thrombocytopenia in otherwise healthy patients. ITP is defined as autoimmune destruction of platelets. Platelet production is typically normal or increased. Its diagnosis requires exclusion of other conditions that cause thrombocytopenia. This disease is roughly analogous to autoimmune hemolytic anemia (Chapter 7). Anti-platelet autoantibodies, often directed against platelet Gp Ib or IIb/IIIa complexes, result in antibody and Fc-dependent platelet destruction by the reticuloendothelial system, primarily in the spleen.

- **ITP has a different natural history in children than in adults:**
 - Children typically have an acute form of the disease, usually following infection, that resolves spontaneously in the majority of cases.
 - The chronic, relapsing form seen in adults can be controlled with therapy but spontaneous remissions are unusual.
- In pregnant women with ITP, maternal autoantibody IgG can cross the placenta and occasionally cause neonatal thrombocytopenia.
- Therapy is guided by severity of the thrombocytopenia and commonly includes observation (in mild cases), glucocorticoids, intravenous immune globulin (IVIG), splenectomy, and various other forms of immune suppression.
- A recent advance in the treatment of ITP has been the introduction of the synthetic thrombopoietic agents eltrombopag and romiplostim, which mimic the effect of thrombopoietin. Although most ITP patients already have normal or increased platelet production, additional production stimulated by these drugs benefits a significant proportion of them.

- **Eltrombopag** is a small molecule thrombopoietin (TPO) receptor agonist that is administered orally once daily.
- **Romiplostim** is a “peptibody”, containing two TPO receptor-binding domains linked to a human immunoglobulin Fc domain. It is administered subcutaneously once per week.
- Both of these drugs increase the risk of reticulin fiber deposition in the bone marrow. To date this has not been associated with clinically apparent adverse effects, but there is concern that the long term use of these agents could lead to marrow fibrosis.

4. Drug-induced immune thrombocytopenia may be caused by many different drugs, most commonly antibiotics (penicillin or sulfonamide-related) and quinine compounds. A careful drug history is critical, and should include herbal remedies, non-prescription medications, and foods (tonic water contains quinine). The mechanism is presumably drug-dependent antibody-mediated destruction of platelets in most cases, similar to drug-induced immune hemolysis (Chapter 7). The practical implication of this association is that during the evaluation of thrombocytopenia, **all non-essential drugs should be stopped**. Recovery generally occurs within 5-7 days after stopping the offending drug. Heparin-induced thrombocytopenia (HIT) is a special case of immune-mediated thrombocytopenia that will be discussed in the thrombosis section.

FYI

Autoimmune disorders and secondary immune destruction- A number of autoimmune disorders such as systemic lupus erythematosus (SLE) can be associated with the development of platelet autoantibodies and thrombocytopenia. Alternatively, high concentrations of circulating antigen-antibody complexes can be adsorbed on the platelet surface, resulting in the non-specific destruction of platelets by the spleen.

5. Thrombocytopenia associated with microangiopathic hemolytic anemia (MAHA)-

Microangiopathic hemolytic anemia (MAHA) is defined by the presence of RBC fragmentation (schistocytes) on the blood smear, varying degrees of anemia, elevated LDH, and usually an elevated reticulocyte count. It is a form of intravascular hemolysis caused by the physical destruction (shearing) of the RBC due to the deposition of thrombi in small vessels, or to diffuse endothelial injury. In the presence of coagulopathy (prolonged coagulation times), this clinical picture suggests disseminated intravascular coagulation (DIC), which is discussed below under acquired coagulopathies. In the absence of coagulopathy, the syndrome of thrombocytopenia and MAHA suggests thrombotic thrombocytopenic purpura (TTP), hemolytic-uremic syndrome (HUS), or diffuse endothelial injury (e.g., malignant hypertension, organ rejection, or vasculitis).

- **Thrombotic thrombocytopenic purpura (TTP)-** This disorder is a complication of acquired (or, very rarely, inherited) severe deficiency (< 5% activity) of the vWF-

cleaving protease ADAMTS-13. It is usually caused by the development of autoantibody inhibitors against ADAMTS-13. Acute episodes are often triggered by infection, pregnancy, medications, or other endothelial injury. **Deficiency of ADAMTS-13 results in the circulation of ultra-large vWF multimers, which predispose to the formation of spontaneous platelet aggregates and the deposition of platelet and vWF-rich thrombi in small vessels. These small vessel thrombi result in microangiopathy and organ dysfunction due to ischemia.** TTP is characterized by thrombocytopenia, MAHA, fever, and dysfunction of various organs, in particular the brain (change in mental status, seizures) and kidneys. In contrast to disseminated intravascular coagulation (discussed below), there is no consumption of clotting factors, and coagulation assays are usually normal. Relapses after treatment are common. Current therapy includes plasma exchange or plasma infusion (plasma contains vWF-cleaving protease), steroids, and other immunosuppressive therapy. *Before plasma exchange became standard therapy, TTP had a mortality rate approaching 90%; with plasma exchange, mortality is generally less than 20%. Thus, prompt recognition and treatment of this clinical syndrome is critical.*

- **Hemolytic-uremic syndrome (HUS)**- shares many of the clinical and pathologic characteristics of TTP, but the kidneys are the dominant organ involved. This syndrome is classically associated with a prodrome of bloody diarrhea resulting from infection with enterohemorrhagic bacteria expressing Shiga-like toxin (e.g., *Escherichia coli* O157:H7). Exposure to Shiga-like toxin appears to trigger endothelial injury, which has a predilection for the renal vasculature. Idiopathic forms of this disease (not associated with a diarrhea prodrome) also occur, frequently associated with mutations in certain complement regulatory proteins.

FYI

Diffuse endothelial injury in a number of conditions—including malignant hypertension, solid organ rejection, bone marrow transplantation, specific chemotherapy drugs, and vasculitis—can cause MAHA.

B. Acquired Defects in Platelet Function

- **Uremia:** results in defective platelet function. The mechanism is unclear but appears to depend on small, dialyzable molecules. This defect responds to dialysis, raising the hematocrit (>30%), and administration of conjugated estrogens or DDAVP.
- **Drugs: the most important (common) drugs that inhibit platelet function are non-steroidal anti-inflammatory drugs (NSAIDs) and aspirin.** These drugs block thromboxane production by the platelet in a reversible (NSAID) or irreversible (aspirin) fashion, resulting in prolongation of the bleeding time. Because aspirin irreversibly inhibits cyclo-oxygenase (COX-1), this anti-platelet effect last up to 7-10 days (life span of platelets). Other important drugs that inhibit platelet function include clopidogrel, which inhibits ADP-mediated platelet activation, and Gp IIb/IIIa antagonists such as

abciximab, tirofiban, or eptifibatide, which inhibit platelet aggregation by blocking fibrinogen binding. These drugs are discussed in more detail in Chapter 11, part 2.

FYI

Myeloproliferative or myelodysplastic disorders (Chapter 11), and in particular essential thrombocythemia (ET), may exhibit platelet defects, particularly at very high platelet counts. These patients may be at risk for either bleeding or thrombotic complications. Myelodysplastic syndromes (MDS) may exhibit quantitative and/or qualitative platelet defects.

C. Thrombocytosis: Causes

- **Reactive-** usually associated with chronic underlying inflammation (e.g., rheumatoid arthritis) or major trauma (e.g., motor vehicle accident). Generally, no therapy is required even at very high platelet counts.
- **Myeloproliferative disorders** (e.g., essential thrombocytosis)- see Chapter 8.
- **Post-splenectomy or asplenia-** associated with Howell-Jolly bodies and mild leukocytosis.
- **Iron deficiency-** resolves with replacement therapy.

D. Acquired Coagulopathies

1. Vitamin K deficiency

Vitamin K is a lipid-soluble substance that is present in many foods (particularly green leafy vegetables), and synthesized by intestinal bacteria. Its function is described in more detail in Chapter 11. **It is required for the production of prothrombin, factors VII, IX, and X, and the anticoagulant proteins C and S. Antagonism of vitamin K-dependent post-translational modification of these factors is the mechanism for the antithrombotic effect of warfarin** (see Chapter 11, part 2). Prolongation of the PT and PTT and (in severe cases) bleeding may occur. Conditions associated with vitamin K deficiency include antibiotic therapy, nutritional deficiency, biliary obstruction (bile salts are necessary for proper absorption of the vitamin), malabsorption syndromes, and ingestion of warfarin or related compounds. Oral or parenteral vitamin K begins to replete coagulation factors within 12-24 hours, but 2-3 days are required for maximal effects. Serious bleeding should be treated with fresh frozen plasma (FFP) or prothrombin complex concentrate (a plasma-derived preparation highly enriched in the vitamin K-dependent clotting factors) at presentation.

Hemorrhagic Disease of the Newborn (HDN)

Newborn babies are vulnerable to bleeding due to Vitamin K deficiency. This is a consequence of poor transport of vitamin K by the placenta and lack of colonization of the newborn gut by bacteria. Furthermore, breast milk is a poor source of Vitamin K. There are 3 distinct presentations: 1) very early – on day one of life, often associated with

intracranial hemorrhage; 2) early – days 1-7, presenting with GI bleeding and ecchymosis in an exclusively breast fed infant; and 3) late – often occurring at 1-3 weeks in an infant with a complicating gastrointestinal illness such as cystic fibrosis, alpha-1 antitrypsin deficiency or diarrhea, associated with intracranial bleeding, bleeding from the umbilical stump, and/or ecchymosis. **To reduce the risk of HDN, all infants receive prophylactic Vitamin K, either by injection or orally, on the first day of life.**

2. Liver disease

Severe liver disease causes decreased synthesis of most coagulation factors including fibrinogen, prothrombin and factors V, VII, IX, X, and XI. Bleeding in patients with liver disease represents a difficult therapeutic problem. Replacement therapy with FFP causes temporary improvement. Most patients do not respond to vitamin K replacement. Local sites of bleeding, including gastritis and esophageal varices may require specific treatment. DIC and hyperfibrinolysis may also complicate acute or chronic liver disease (see below).

3. Disseminated intravascular coagulation (DIC)

DIC is a syndrome in which thrombin and plasmin are generated at a rate that exceeds the ability of their natural inhibitors (antithrombin and α_2 -antiplasmin) to neutralize them. It is usually caused by exposure of blood to excessive amounts of tissue factor. In contrast to the normal localized coagulation response, thrombin and/or plasmin activity is present in the systemic circulation, and generation of fibrin and platelet activation occur in a disorganized manner. The consequences of this excessive, systemic generation of thrombin and plasmin include consumption of coagulation factors and platelets, depletion of inhibitors, bleeding, deposition of fibrin in small vessels with resulting microangiopathy, and varying degrees of organ dysfunction. The degree to which the fibrinolytic system is activated in DIC is variable, but when present, excessive fibrinolysis increases the risk of bleeding.

It should be emphasized that **DIC is a syndrome and not a specific diagnosis**. It may be associated with a variety of underlying diseases, including:

- Infection
- Solid tumors, particularly adenocarcinomas
- Leukemia
- Obstetric complications
- Acute hemolytic transfusion reactions (see Chapter 6)
- Severe liver disease
- Massive trauma
- Surgery
- Shock

Diffuse endothelial injury appears to play an important role in many cases of DIC and is a major contributor to the associated organ dysfunction. DIC may be acute or chronic, and

the severity may vary greatly. Chronic DIC is particularly common in patients with solid tumors. Laboratory findings in DIC include:

- Thrombocytopenia
- Prolonged PT/INR and PTT
- Elevated FDP and D-dimer
- Decreased fibrinogen
- Red cell fragmentation on the blood smear

Treatment of the underlying condition is the major therapeutic approach.

Replacement of depleted clotting factors with FFP and platelet transfusion may be necessary in more severe cases.

4. Acquired coagulation inhibitors

These are usually circulating immunoglobulins of the IgG class. These inhibitors can be alloantibodies that arise in the context of factor replacement therapy for patients with hemophilia, or autoantibodies that arise spontaneously without a pre-existing coagulation defect, usually in elderly patients. Laboratory evaluation demonstrates a failure to correct the prolonged coagulation time (usually PTT) in mixing studies. These inhibitors are most common in severe hemophiliacs, presumably because these patients are more likely to recognize the replaced factor as a foreign antigen. In hemophilia A or B, these inhibitors neutralize the clotting factor given to treat the disease, and can result in life-threatening bleeding. **Spontaneous coagulation inhibitors are usually directed against factor VIII (acquired hemophilia) and are often associated with severe or fatal bleeding.**

FYI

Patients with factor VIII inhibitors can be treated acutely with a “bypassing” agent such as recombinant factor VIIa, or an activated prothrombin complex concentrate preparation that contains all of the vitamin K-dependent clotting factors in both zymogen and protease forms. Patients with spontaneous inhibitors are usually given immunosuppressive treatment as well.

FYI

Non-Thrombocytopenic Purpura (Vascular Purpura)

Although the presence of unexplained purpura (petechiae or ecchymoses) often triggers evaluation for a hemostatic defect, it is important to understand that a number of other conditions can cause egress of red blood cells from vessels. Increased transmural pressure can be either an acute (Valsalva with coughing, vomiting), or chronic (venous stasis) etiology. Decreased mechanical strength of the microcirculation can result from an inherited (Ehlers-Danlos syndrome) or acquired connective tissue or vessel defects (scurvy, infiltration by amyloid, glucocorticoids, aging). Endothelial damage may result from infection (Rickettsial, viral), trauma including factitious purpura (usually involving a suction device applied to the skin), embolism (cholesterol, fat), and allergy or inflammation (serum sickness, vasculitis).

CHAPTER 13

THROMBOTIC DISORDERS

Key Concepts:

Pathophysiology, diagnosis, and treatment of hereditary and acquired thrombotic disorders.

Learning Objectives:

1. Describe the typical characteristics of venous and arterial thrombi.
2. List the components of Virchow's triad, and describe the contribution of each component to the pathophysiology of thrombosis.
3. List and compare the major clinical risk factors for arterial and venous thrombosis.
4. Compare and contrast the approach to treatment for a patient with arterial thrombosis to a patient with venous thrombosis.
5. List five hereditary disorders that increase the risk of venous thrombosis, and briefly describe how the genetic defect in each condition increases the risk of thrombosis.
6. Describe the major diagnostic tests for venous thromboembolism.
7. Describe the most important laboratory findings and clinical features associated with antiphospholipid syndrome.
8. Describe the indications, mechanism of action, major complications, and suggested laboratory monitoring of therapy with unfractionated heparin and low molecular weight heparin.
9. Describe the pathophysiology of heparin-induced thrombocytopenia.

I. Pathophysiology and Genetics of Thrombotic Disorders

A. Definitions/Descriptions

- **Thrombosis**- a term that describes the pathologic process in which intra-luminal (or intra-cardiac) thrombus interrupts the arterial supply or venous drainage of a limb or organ system. Thrombosis occurs through the same biochemical pathways as coagulation and platelet activation/aggregation, suggesting defective regulation of the response.
- **Arterial thrombosis may induce ischemic necrosis of the particular organ or limb. Arterial thrombi are typically platelet rich** (“white clot”), formed under conditions of high shear stress (rapid flow) with underlying endothelial abnormalities (e.g., atheromata), and are generally occlusive, leading to infarction. Examples include myocardial infarction, thrombotic (occlusive) stroke, and mesenteric ischemia. Arterial thrombi can form emboli, portions of the clot that break off and travel into the distal circulation. The most common example is embolic stroke from an intracardiac thrombus caused by atrial fibrillation, valvular disease, or severe left ventricular dysfunction.
- **Venous thrombosis can induce edema, swelling, pain, and inflammation in the affected limb. Most commonly, venous thrombosis involves the deep venous system in the lower extremities or pelvis. Venous thrombi are fibrin rich** (“red clot”), occur in areas of venous stasis (see Virchow’s triad below), and in large veins tend to originate in valve cusps. Thrombus formation is generally cyclic, with layers enriched for fibrin and red blood cells. Deep venous thrombosis (DVT) can result in **pulmonary embolism (PE)**, in which all or part of the thrombus breaks free and follows the venous return through the right side of the heart to lodge in a pulmonary artery. Massive pulmonary embolism can cause hypoxemia, shock, and death.
- **Mural thrombus** is a term used for a clot that forms on the wall of an underlying structure. This is most frequently seen in the heart and aorta.
- **Paradoxical embolus** refers to an embolus originating in a vein that bypasses the lung and enters the arterial circulation via a patent ductus arteriosus or atrial septal defect.
- **Thrombus evolution:** thrombi may undergo resolution (complete healing), embolization (see above), or organization (recanalization and/or incorporation into the vessel wall).

B. Pathophysiology of Arterial Thromboembolism

Development of arterial thrombosis is closely linked to atherosclerotic vessel disease, making it difficult to discern risk factors for arterial thrombosis separately from factors that predispose to the underlying vessel disease.

The development of arterial atherosclerotic plaques is mediated by inflammation and endothelial injury. Increased expression of endothelial adhesion molecules (VCAM-1)

- recruits inflammatory cells
- stimulates cytokine release, lipid oxidation, or uptake by monocyte/macrophages
- stimulates smooth muscle proliferation

Plaque formation results in turbulent flow and increased shear stress, triggering platelet activation and thrombus formation on complicated plaques. Loss of the anticoagulant (thrombomodulin/protein C), antiplatelet (nitric oxide, ecto-ADPase), and pro-fibrinolytic properties of normal endothelium (Chapter 8) likely contributes to this process.

Underlying atherosclerosis is associated with increased age, smoking, hypertension, obesity, hypercholesterolemia, diabetes mellitus, family history of heart disease, and sedentary life style. Additional risk factors for the development of arterial thrombotic events in this context include:

- **Inflammation** is associated with increases in **acute phase proteins** such as fibrinogen, factor VIII, and C-reactive protein (CRP), and the **activation of coagulation**. Inflammatory cytokines (TNF α , Il-1 β , and Il-6) activate coagulation via a number of mechanisms including the induction of monocyte tissue factor activity, release of tissue factor-bearing microvesicles, and down-regulation of anticoagulant (thrombomodulin) and fibrinolytic (increased PAI-1) properties of the endothelium. The precise mechanisms by which the inflammatory and coagulation responses interact are active areas of investigation.
- Risk factors associated with **both** arterial and venous thrombosis include hyperhomocysteinemia, heparin-induced thrombocytopenia, and antiphospholipid syndrome/lupus anticoagulants (discussed with venous thrombosis).

Clinical presentation depends on the specific vascular bed involved and the acute vs. chronic nature of the arterial obstruction. Common clinical problems involve coronary arteries (angina and MI), carotid and intracerebral arteries (TIA and stroke), and peripheral arterial beds (claudication and gangrene). **Formation of thrombus on an underlying atherosclerotic plaque is often the final event** in clinical presentations such as myocardial infarction.

Specific clinical syndromes are covered in your cardiovascular and neuroscience courses.

C. Pathophysiology of Venous Thromboembolism (VTE)

Venous thromboembolism (VTE) is a multicausal disease in which the interaction of acquired clinical and genetic risk factors determines the likelihood of occurrence. **In contrast to bleeding disorders, VTE is a multigenic disorder** (similar to hypertension or diabetes). Since the etiology is multigenic, we consider inheritance of defective alleles as genetic risk factors for development of thrombosis, not disease states per se.

Factors involved in the development of venous thrombi are summarized in Virchow's triad:

- **venous stasis**- Decreased mobility, intrinsic or extrinsic vessel obstruction
- **endothelial/vessel wall abnormalities**- trauma, surgery, atherosclerosis, vasculitis, etc.
- **intrinsic "hypercoagulability" of the blood**
 - **Inherited prothrombotic traits** such as Factor V Leiden or antithrombin deficiency, as discussed below.
 - **Acquired factors:** Inflammation, lupus-type anticoagulant/antiphospholipid syndrome, consumption/loss of natural anticoagulants (DIC or nephrotic syndrome), increased platelet reactivity (myeloproliferative disease, discussed in Chapter 11, or heparin-induced thrombocytopenia, discussed below).

D. Clinical (Acquired) Conditions Associated with VTE

A variety of clinical conditions affect Virchow's triad and predispose to the development of venous thromboembolism. The lists below provide a way to think about clinical risk factors for VTE, although many risk factors have effects in more than one category. For example, surgery may result in both immobility and endothelial injury. Clinical risk factors may be **transient** (pregnancy) and/or **reversible** (estrogen therapy), or represent **chronic, ongoing** conditions (age, obesity, malignancy). Chronic, ongoing clinical risk factors increase the relative risk for recurrent VTE. Thromboembolic events that occur in the absence of clinical risk factors are referred to as idiopathic or unprovoked VTE. **Up to 30% of patients with idiopathic VTE may suffer a recurrence, suggesting that many such patients have undetected (possibly inherited) chronic risk factors for venous thrombosis.**

1. Venous Stasis

- bed rest/immobility (e.g., stroke, lower extremity casts, long distance travel)
- obesity
- congestive heart failure (CHF)
- venous obstruction/post-phlebotic syndrome
- advanced age

2. Endothelial/vessel injury

- trauma
- surgery/general anesthesia (especially hip and knee fracture/replacement)
- pregnancy (especially post-partum)
- smoking

3. Intrinsic hypercoagulability

- lupus anticoagulant/antiphospholipid antibodies
- pharmacologic estrogen doses (oral contraceptives, hormone replacement)
- malignancy (especially adenocarcinomas)
- heparin-induced thrombocytopenia (HIT)
- myeloproliferative disorders (polycythemia vera, essential thrombocythemia)

- nephrotic syndrome
- disseminated intravascular coagulation (DIC)

E. Commentary on Some Acquired Risk Factors

1. Lupus anticoagulants/antiphospholipid antibodies

- **Lupus anticoagulants (LAC) are phospholipid-dependent antibodies that inhibit *in vitro* coagulation. The nomenclature is somewhat misleading because they *do not require an underlying diagnosis of systemic lupus erythematosus (SLE), and are associated with both arterial and venous thrombosis in vivo (not bleeding).* Typically, they prolong the APTT (or other phospholipid-dependent clotting assay) in an inhibitor pattern (mixing studies do not correct), and are neutralized by the addition of excess lipid or platelets.**
- **Antiphospholipid antibodies (APA)** represent a larger category of phospholipid-dependent antibodies that mostly do not affect *in vitro* coagulation assays (lupus anticoagulants are a subset of these antibodies). These antibodies may be directed against a variety of epitopes, including **cardiolipin** and **β_2 -glycoprotein I**. The detection of these antibodies is common enough in the general population that their pathologic significance is unclear, unless present in high titers, in association with other autoantibodies, or in patients with other evidence of the antiphospholipid antibody syndrome.
- **Antiphospholipid antibody syndrome:** the presence of persistent antiphospholipid antibodies (LAC or other APA) in association with a syndrome of arterial and/or venous thrombosis, recurrent fetal wastage, and autoimmune thrombocytopenia (usually mild). The syndrome may occur in the context of underlying SLE or with no associated condition (primary antiphospholipid syndrome).

2. Heparin-induced thrombocytopenia (HIT)

Heparin therapy **commonly** causes a mild (15-30% decrease), early onset (1-3 days) thrombocytopenia that is not immune-mediated, and has no pathological significance. **In contrast, heparin rarely causes a moderate to severe (greater than 50% reduction), late onset (5-7 days) thrombocytopenia due to development of a heparin-dependent antibody.** Low molecular weight heparin (LMWH) is associated with a lower risk of HIT relative to unfractionated heparin (UH).

Heparin-induced thrombocytopenia (HIT) is associated with an estimated 50% incidence of arterial and venous thrombosis within 30 days and up to 20% in-hospital mortality.

Untreated HIT can cause progressive life and limb-threatening thrombosis.

FYI

Mechanism: Platelet factor 4 (PF4) is a platelet protein that binds tightly to heparin chains. PF4 undergoes a conformational change upon heparin binding, exposing “neoepitopes” that trigger antibody formation versus the platelet factor 4 (PF4)-heparin complex. Formation of this IgG-PF4-heparin complex on the platelet surface causes Fc receptor-dependent platelet activation and release of additional PF4 from α -granules. This antibody can also recognize PF4-heparan sulfate complexes on the endothelial surface. The marked platelet activation results in moderate thrombocytopenia (due to consumption) and may trigger fulminant arterial and/or venous thrombosis.

- **Therapy:** immediately discontinue **all** heparin (including IV flushes) and consider alternative anticoagulation argatroban or fondaparinux The latter drug, although chemically similar to heparin, does not cross-react with the antibodies that cause HIT. There is insufficient data to support administration of an oral thrombin or factor Xa inhibitor in HIT, although in principle these drugs should be effective in this condition as well.

3. Disseminated intravascular coagulation (DIC) and fibrinolysis – see Chapter 12

4. Thrombotic thrombocytopenic purpura (TTP) and hemolytic uremic syndrome (HUS) – see Chapter 12

5. Myeloproliferative/bone marrow disorders (see chapter 8)

- **Polycythemia vera-** is associated with increased incidence of venous thrombosis.
- **Essential thrombocythemia-** the elevated platelet count can be associated with both bleeding and thrombotic complications. Venous thrombosis and microvascular arterial events are most common, including digital ischemia. Must be differentiated from **reactive thrombocytosis**, which generally poses little to no risk.

F. Genetic Risk Factors for VTE

Currently known inherited risk factors generally represent defects in the natural anticoagulant pathways (antithrombin, protein C and S, Factor V Leiden) or elevated coagulation factors levels (e.g., prothrombin G20210A) that regulate thrombin generation. Each of these risk factors has a modest individual contribution to the overall risk of venous thromboembolism. Thus, even in patients with underlying genetic predisposition, thrombotic events are generally triggered by addition of one or more acquired factors (surgery, pregnancy, immobility). The relative risk for venous thrombosis compared to the general population is increased up to 20 fold in some families with hereditary antithrombin, protein C, or protein S deficiency. The relative risk in individuals with factor V Leiden or prothrombin mutation is increased ~2-5 fold.

1. **Antithrombin (AT, also called antithrombin III) deficiency**- rare (1/5,000 blood donors), autosomal dominant condition in which AT activity levels are about 50% of normal. The homozygous state (complete absence of antithrombin in the blood) has never been described and is probably not compatible with life.
2. **Protein C deficiency**- more common (1/350 blood donors), autosomal dominant condition in which protein C activity is about 50% of normal. The homozygous state (complete or near-complete absence of protein C) results in a severe thrombotic disorder beginning in infancy (neonatal purpura fulminans).
3. **Protein S deficiency**- rare autosomal dominant condition, no good estimates of prevalence. Homozygous state also associated with neonatal purpura fulminans.
4. **The Factor V Leiden polymorphism is the most common (5% of Caucasian population) autosomal dominant condition that is associated with a 4-5 fold increase in the relative risk of venous thromboembolism.** Homozygotes have substantially higher risk than heterozygotes (perhaps 50 times higher than general population). Unlike antithrombin, protein C and protein S deficiencies, which are associated with many different mutations, the molecular defect in factor V Leiden is a single base change in the factor V gene that eliminates a protein C cleavage site in factor Va, resulting in **activated protein C resistance** and defective termination of coagulation by activated protein C.
5. **Prothrombin G20210A polymorphism** is a relatively common (2% of the general population), autosomal dominant condition that is associated with 15-30% higher levels of prothrombin antigen in the plasma. This is due to a single base change in the 3' untranslated region of the prothrombin gene. The specific mechanism by which it increases prothrombin expression is unknown.

Genetic/Acquired Risk Factors

6. **Hyperhomocysteinemia**- is a risk factor for both venous and arterial thrombosis. Severe elevations result from homozygous cystathionine synthetase deficiency (homocystinuria, a rare congenital disease). Mild elevations are much more common, and may result from other inherited or acquired defects in homocysteine metabolism. The mechanism is unclear, but may involve induction of endothelial procoagulant activity by homocysteine and related compounds. Lowering homocysteine levels (for example, by administration of folate and B₁₂) has not lowered thrombotic risk, at least in the short term, in several clinical trials.
7. **Elevated factor VIII levels**- some instances seem to be familial, but increased levels are also commonly associated with inflammatory states.

G. Summary- Pathophysiology and Risk Assessment in VTE

Approximately 250,000-300,000 cases of venous thrombosis are diagnosed annually in the U.S., with the incidence increasing sharply with age. Major complications include the post-phlebitic syndrome (chronic, sometimes disabling, leg swelling and pain due to venous stasis) and sudden

death from pulmonary embolism. Anticoagulant therapy given to prevent recurrent venous thromboembolism has significant clinical benefits, but is also associated with an increased risk of bleeding complications. The risk of **recurrent** venous thromboembolic disease varies significantly depending on the clinical and genetic risk factors present in a particular patient. Clinical risk factors may be transient or reversible (surgery, estrogens), or alternatively represent chronic conditions (congestive heart failure, venous insufficiency). Patients with idiopathic/unprovoked VTE, or those with known irreversible clinical risk factors, are at the highest risk for recurrent events. Genetic risk factors for VTE obviously represent chronic thrombophilic conditions. The heterogeneity in thrombotic risk among families with single gene defects, and the lack of established risk factors in the majority of thrombophilic patients, suggests that additional unknown genetic factors are involved in the development of thrombosis in many cases. The ability to select patients with a high likelihood of recurrent venous thromboembolism for long-term anticoagulation would greatly improve the risk: benefit ratio of therapy.

Caveats regarding the laboratory evaluation of hypercoagulable states. The multigenic etiology of venous thromboembolism and our incomplete knowledge of the risk-associated alleles significantly limit the ability to perform global genetic risk assessment. Thus, **laboratory evaluation of hypercoagulable states does not contribute to the clinical management of most patients.** Such testing may be helpful in selected patients presenting at a young age, and/or with a significant family history. Antithrombin, protein C, and protein S deficiency are associated with the highest risk of recurrent VTE. However, in a patient with VTE associated with a transient or reversible risk factor (for example, surgery or pregnancy), the risk of recurrence tends to be low even in the presence of such thrombophilic conditions and long-term anticoagulant therapy is generally not justified. Conversely, in patients who have unprovoked VTE, the risk of recurrence is relatively high regardless of whether or not there is laboratory evidence of thrombophilia. It is also important to realize that although the thrombophilic mutations and polymorphisms described above are associated with significant increases in the *relative* risk of VTE, the *absolute* risk of thrombotic events is generally not high enough to warrant anticoagulant therapy in asymptomatic carriers.

II. Diagnosis of Venous Thromboembolism (VTE)

The manner in which patients present with venous thromboembolic events has implications for the relative risk of recurrence (idiopathic versus triggered events), and therefore for decisions regarding the recommended duration of anticoagulant therapy. The signs and symptoms of venous thromboembolism are sufficiently non-specific that **objective testing to demonstrate the presence of thrombi is critical.** Testing may include duplex Doppler ultrasonography or contrast or MR venography for DVT, and ventilation/perfusion lung scan or spiral CT for PE. The plasma D-dimer level is a sensitive but not specific indicator of VTE. A normal D-dimer level makes VTE unlikely, but in a patient with an elevated D-dimer level VTE should still be confirmed by one of the imaging studies listed above.

III. Overview of therapeutic indications for Antithrombotic therapy

See part 2 of this chapter for discussion of individual drugs.

A. Standard Treatment of Venous Thromboembolic Disease

- **Uncomplicated DVT/PE**- standard of care is at least 5 days of heparin (UH or LMWH), overlapping with warfarin to achieve a target range INR 2.0-3.0. Oral thrombin and Xa inhibitors, discussed in chapter 11, are potential alternatives to warfarin.
- **Duration of therapy**- 3-6 months for first event
- **“Idiopathic” DVT**- occurring in the absence of precipitating clinical factors, and has a higher incidence of recurrent events. Often treated for 6-12 months, but optimal duration of therapy remains unclear and some patients may benefit from long-term anticoagulation.
- **Vena cava filters**- mechanical device inserted into the IVC that traps emboli coming from the legs. Used to prevent pulmonary embolism in patients with strong contraindications to anticoagulation, or for patients who have recurrent events while on therapeutic anticoagulation.

B. Prophylaxis of Venous Thromboembolism

Many hospitalized patients are at risk for venous thromboembolism. At highest risk are those having orthopedic procedures such as hip or knee replacement and those with major trauma, in whom the risk of VTE may exceed 50%. General, thoracic and gynecologic surgery patients, and non-ambulatory patients with acute medical illness and stroke are also at significant risk for VTE. It has become standard practice to treat such patients to lower their risk of VTE. The most effective form of prophylaxis is anticoagulation, but the benefits of anticoagulant prophylaxis must be weighed against the risk of bleeding associated with such treatment. Treatment modalities include:

- Heparin, low molecular weight heparin and fondaparinux, given subcutaneously at doses lower than those typically used to treat established VTE.
- Mechanical prophylaxis with intermittent pneumatic compression devices and graded compression stockings applied to the legs. Mechanical methods are somewhat less effective than pharmacologic prophylaxis, but may be preferred in patients at high risk for bleeding.
- Oral agents such as dabigatran (a thrombin inhibitor) and rivaroxaban (a factor Xa inhibitor) have also been shown to be effective prophylactic agents. They are associated with bleeding risks comparable to heparin and related drugs.

B. Anticoagulation for Mechanical and Prosthetic Heart Valves

- **Mechanical valves**- lifelong risk of thromboembolism, target INR 2.5 to 3.5. Mitral valve position is higher risk than the aortic position.
- **Bioprosthetic valves**- increased risk of thromboembolism for first 3 months, target INR 2.0-3.0

C. Anticoagulation for Atrial Fibrillation

- Chronic atrial fibrillation is associated with a significantly increased risk of thromboembolic stroke.

- Large clinical trials have demonstrated that warfarin is superior to aspirin for prevention of stroke in most patients.
- A subset of low-risk patients with atrial fibrillation who do not have underlying structural heart disease (valvular or left ventricular dysfunction), hypertension, or diabetes do equally well on aspirin as compared to warfarin.
- Dabigatran and rivaroxaban are now approved for stroke prevention in chronic A-Fib, and appear to have efficacy and safety at least as good as warfarin.

D. Antiplatelet Agents for primary and secondary prevention of cardiovascular events

1. **Aspirin (ASA)**- irreversibly inhibits platelet cyclo-oxygenase (COX-1), blocking the production of thromboxane A₂, an important mediator of platelet activation (Figure 13.2). This anti-platelet effect lasts for 5-7 days (similar to platelet lifetime). Cheap and effective therapy in a number of clinical syndromes (see Table 13.1). Aspirin is the standard of comparison for other platelet agents. See also chapter 11.

Table 13.1 Use of ASA in thrombotic disorders (from Patrono, C. *Chest* 2004;126:234S-264S)

*

| Disorder | Minimum Effective Daily Dose (mg) |
|---------------------------------|--|
| Men at high cardiovascular risk | 75 |
| Hypertension | 75 |
| Stable angina | 75 |
| Unstable angina* | 75 |
| Acute MI | 160 |
| TIA and ischemic stroke* | 50 |
| Severe carotid artery stenosis* | 75 |
| Acute ischemic stroke* | 160 |

*Higher doses have been tested in other trials and not found to confer any additional benefit.