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Essentials in Hematology and Clinical Pathology

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Foreword

"Some books are to be tasted, others swallowed and some few to be chewed and digested," said Francis Bacon. This book, for which I am delighted to write the Foreword, has to be not merely tasted, chewed, swallowed and digested but also absorbed and assimilated for best results!

Clarifying concepts and inculcating the foundational tenets in hematology and clinical pathology are daunting tasks for any teacher. The teachers who have authored this book have admirably captured the core of the subject capping that with lucid explanations. The authors have expertise not only in the subject but also in the art of teaching. Willingness to share and the ability to convey convincingly are the veritable attributes of the authors. It is this wisdom of experience and expression that has percolated into this book. A book of this substance and style was long overdue and it could not have been timelier. I am certain that the contents of the book will mellow with each succeeding edition in the years to come. Let me congratulate and commend the authors for the pains they took to bring out a book, which deserves much more than just having a look.



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Preface

Hematology is one of the rapidly expanding fields of medicine. The extensive amount of time and effort spent by the students in going through volumes of the hematology textbooks encouraged us to write this comprehensive book. Understanding hematology requires insight into the applied physiology and the pathophysiologic basis of these diseases in the context of relevant clinical features which is often neglected. *Essentials in Hematology and Clinical Pathology* emphasizes on building a strong foundation of both concepts. Clinical pathology section of the book deals with both hematological and nonhematological laboratory investigations done in routine practice.

The book has been organized into four sections namely ***Disorders of Red Cells, Disorders of White Cells, Disorders of Hemostasis*** and ***Clinical Pathology.***

Essentials in Hematology and Clinical Pathology also features:

- Molecular basis of common hematological disorders
- About 135 illustrations, 27 photomicrographs, 18 photographs and 146 tables have been provided to facilitate easy and quick learning
- A summary of the important points at the end of each chapter
- Essay questions, short note questions and 351 MCQs to encourage self evaluation of the important concepts by the students
- Recent WHO classification (2008) of *Tumors of Hematopoietic and Lymphoid Tissues.*

We hope that the textbook will provide a foundation to the students in hematology and clinical pathology and improve their understanding of the subject.

Ramadas Nayak
Sharada Rai
Astha Gupta

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We also wish to thank all our family members including Smt Rekha Nayak, Dr Narendra Shetty, Dr Amit Gupta and Master Jagrath who have patiently accepted our long preoccupation with this work. Our sincere gratitude to our parents who will always remain our guiding light. We are grateful to all our friends, undergraduate and postgraduate students who have inspired and supported us.

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SECTION 1

Disorders of Red Cells

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CHAPTER 1 Introduction

CHAPTER OUTLINE

- ❖ Definition
- ❖ Hematopoiesis

DEFINITION

Hematology is defined as the **study of** normal and pathologic aspects of blood and **blood cells.**
Hematopoiesis (hemopoiesis) is the continuous, regulated process of blood cell production or formation.
Hematopoietic (hemopoietic) System: It consists of all organs and tissues involved in hematopoiesis, and these are divided into **myeloid tissue and lymphoid tissue.** The pluripotent **hematopoietic stem cell (HSC)** is the progenitor of all the cells in blood and gives rise to cells of both myeloid and lymphoid system.

1. **The myeloid tissue** consists of bone marrow (medullary cavity) and the cells derived from it, which include
   - **Red blood cells** (RBCs/ erythrocytes)
   - **White blood cells** (WBCs/leukocytes, except lymphocytes): WBCs consist of:
     - Granulocytes: Neutrophils, eosinophils and basophils are collectively called granulocytes because of their different types of cytoplasmic granules. However, the term granulocyte is often referred to only neutrophils.
     - Monocytes
     - Lymphocytes (even though included under WBCs; they are lymphoid derived).
   - **Platelets** (thrombocytes)

   Compensatory hyperplasia: In adults during pathological states, whenever there is an increased demand for blood cells, the bone marrow undergoes compensatory hyperplasia. This results in replacement of the fatty marrow by hematopoietic tissue.
   - **Extramedullary hematopoiesis:**
     - Normally, the cells of the myeloid lineage arise in the central bone marrow (medullary cavity). If the increased demand of blood cells is not met with compensatory hyperactivity of marrow alone, hematopoietic islands appear in liver and spleen (resulting in hepatosplenomegaly) and even in lymph nodes.
     - The myeloid lineage blood cells arising outside the marrow elsewhere in the body are designated as **extramedullary hematopoiesis (agnogenic myeloid metaplasia)**.

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2. **The lymphoid tissue** consists of thymus, lymph nodes and spleen. The common lymphoid progenitor cell gives rise to B cell, T cell and natural killer (NK) cell precursors. They mature to form respective lymphoid cells.

The above division of hematopoietic elements as myeloid and lymphoid tissue is mainly for understanding their pathology. It is not always possible to draw clear demarcation between the diseases affecting them.

Functions of Blood Cells

Formed elements of blood are red cells, white cells and platelets. The main functions of blood cells are presented in Table 1.1.

Table 1.1: Main functions of formed elements of blood

Type of blood cell	*Main function*
Red blood cells	Delivery of oxygen to tissues
White blood cells	Defence against infectious organisms
Lymphocytes	Immune regulation
Platelets	Hemostasis

- As the mature blood elements become old, they are destroyed and constantly produced to maintain normal peripheral blood cell counts.
- In addition, each cell line has the ability to respond appropriately to increased demand like; increased red cell production after blood loss, leukocytosis during infection, wound healing and increased platelet production during chronic bleeding.
- Despite the wide functional diversity of blood cells, all myeloid and lymphoid cells originate from common precursor; pluripotent **hematopoietic stem cells (HSC)**.

HEMATOPOIESIS

Sites of Hematopoiesis

Different sites of hematopoiesis during various phases of life are shown in Table 1.2.

Table 1.2: Different sites of hematopoiesis during various phases of life

Phase	*Period*	*Site*
Yolksac (mesoblastic) phase	First 3 months of gestation	Yolk sac
Hepatic phase	4- 9 months of gestation	Liver (chief site till birth), spleen (minor site)
Medullary (myeloid) phase	By 3rd week after birth up to puberty	Bone marrow throughout the skeleton
	Adults	Active marrow is limited to the ends of long bones and flat bones

Terminology Used in Hematopoiesis

The meaning of the terms used with reference to hematopoiesis is shown in Table 1.3.

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Table 1.3: Different terminologies used with regards to hematopoiesis

Terminology	
Pluripotent	Ability to generate all mature hematopoietic cells
Multipotent	Ability to produce a limited range of differentiated cell lineages appropriate to their location
Unipotent	Restricted ability to differentiate and generate one specific cell type

Normal Development of Blood Cells (Hematopoiesis)

The hematopoietic system is a hierarchy of cells in which pluripotent hematopoietic stem cells proliferate and differentiate. After several steps, HSCs finally give rise to mature blood cells. This hierarchy (Figs 1.1 and 1.2) consists of:

1. Hematopoietic stem cells
2. Progenitor cells
   a. Multipotent progenitor cells
   b. Committed (unipotent) progenitor cells
3. Precursor cells
4. Maturing and mature cells.

Hematopoietic Stem Cells (HSC)

These are small, undifferentiated mononuclear cells that can generate all the blood cell lineages.

HSCs possess two fundamental properties:

- **Self -renewal:** HSCs are capable of cell division to give rise to more stem cells.
- **Differentiation:** HSCs can differentiate and give rise to two kinds of lineage-specific multipotent **progenitor** cells, the **common myeloid** and the **common lymphoid progenitors.**

Note: Apart from blood cells, the stem cells may be able to differentiate into diverse tissue types (e.g. neuronal, muscle, liver, vascular cells). This change in the differentiation of a cell

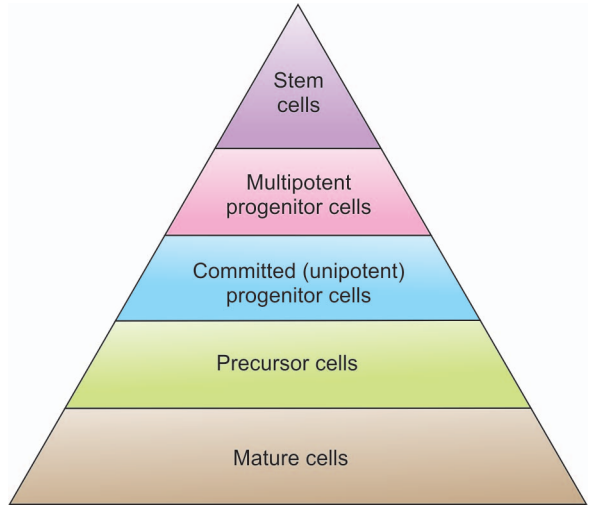


Fig. 1.1: Cell hierarchy in hematopoiesis

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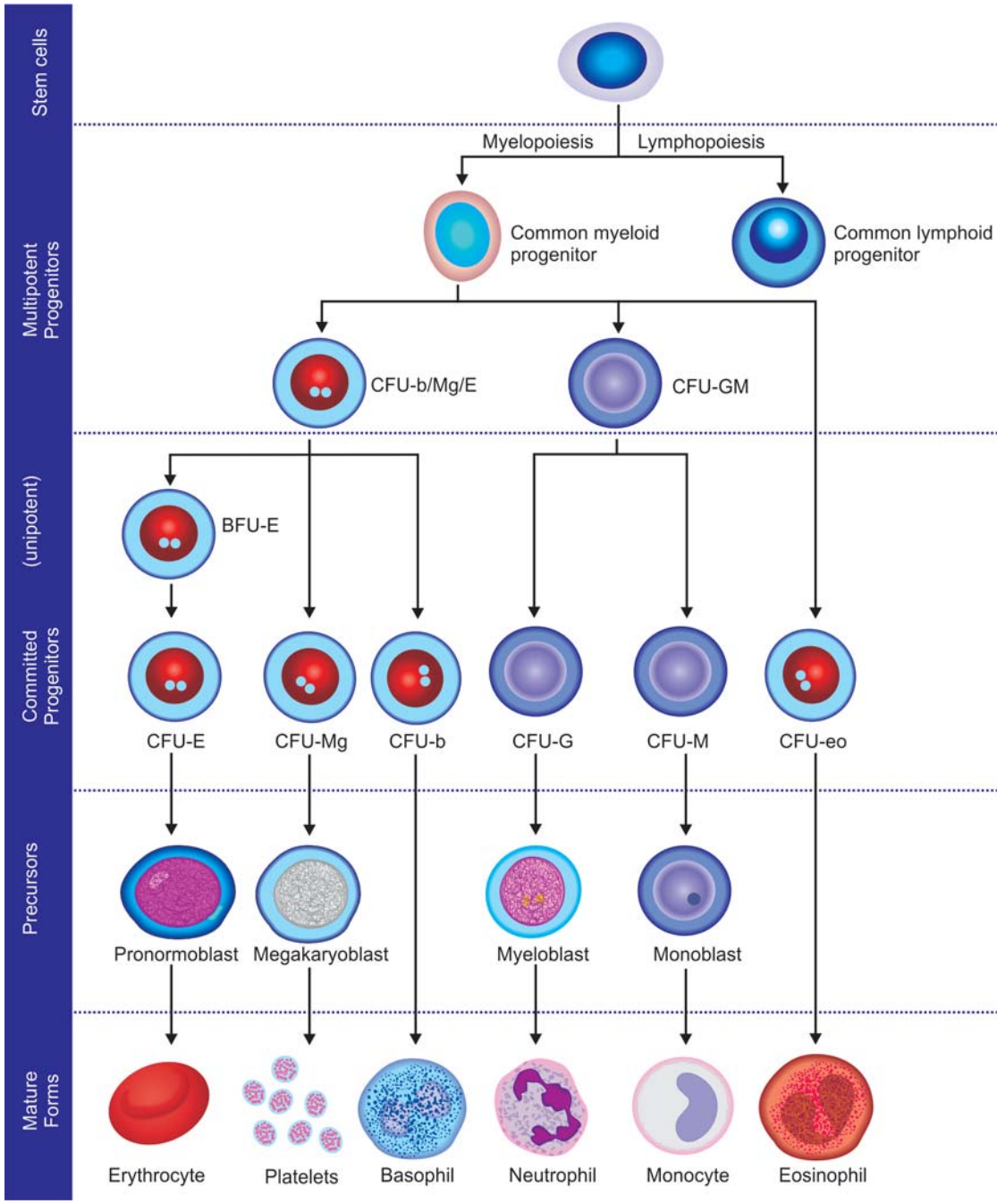


Fig. 1.2: Different stages of hematopoiesis. BFU, burst forming unit. CFU, colony forming unit

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from one type to another is known as **transdifferentiation**, and the capacity of a cell to transdifferentiate into diverse lineages is referred to as **developmental plasticity**.

Progenitor Cells

Upon commitment to development, the HSCs enter the next compartment known as **progenitor cell compartment**. This compartment consists of mainly 2 types of cells.

- **Multipotent progenitor** (lineage specific) **cells**
- **Committed (unipotent) progenitor cells**

Both multipotent and unipotent cells in the bone marrow possess the ability to give rise to clones (groups) composed of specific kinds of mature cells when grown in culture and are called as **colony forming units** (CFU).

Multipotent progenitor cells: These are of 2 types namely: common myeloid progenitor (CMP) and common lymphoid progenitor (CLP).

- **Common myeloid progenitors (CMP)** further divide to produce mainly 2 types of multipotent progenitor cells with restricted differentiation than CMP. They are:
  - CFU b/Mg/E (multipotent) cells which give rise to 3 types of committed (unipotent) progenitor cells.
    - BFU-E (burst-forming unit-erythrocyte) cells and subsequently CFU-E (colony-forming unit-erythrocyte) cells. CFU-E is most sensitive to the action of erythropoietin.
    - CFU-Mg (colony-forming unit-megakaryocyte) cells
    - CFU-Baso (colony-forming unit-basophil) cells
  - CFU-GM (multipotent) cells which differentiate into 2 types of committed (unipotent) progenitor cells.
    - CFU-G (colony-forming unit-granulocyte) cells, precursors of neutrophils
    - CFU-M (colony-forming unit-macrophage) cells, precursors of monocytes and macrophages.

    Note: Committed progenitor cells for eosinophils are produced from CMP CFU-Eo (colony-forming unit-eosinophil) cells.
- **Common lymphoid progenitor (CLP)** cell in turn gives rise to two **progenitor cells.**
  - **Pro T /NK cells:** They give rise to two types of committed (unipotent) progenitor cells.
    - **Pro-T cells** which differentiate into T cells.
    - **Pro-NK cells** which differentiate into NK cells.
  - **Pro B cells:** They give rise to one type of committed progenitor cells.
    - **Pre-B cells** then differentiate into B cells.

    Lymphocyte development is discussed in Chapter 29.

    Morphologically, the progenitor (both multipotent and unipotent) cells and stem cells cannot be distinguished from one another on morphological appearance or cytochemistry except by immunological techniques.

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Precursor Cells

When the immature hematopoietic cell acquires recognizable morphological, cytochemical or immunological feature of a single lineage, it is called as precursor cell. The next step in hematopoiesis is maturation of unipotent progenitor cells to precursor cells. The earliest morphologically recognizable precursor cell of each lineage is termed by adding the suffix "**blast" to the type of lineage** (e.g. lymphoblast to lymphoid lineage).

Mature cells

The precursor cells finally give rise to mature blood cells, which are released from the marrow into the circulation.

Regulation of Hematopoiesis

The growth of different hematopoietic cells is regulated by a number of hematopoietic growth factors, which in general are called cytokines.
Most important growth factors acting on various cells are mentioned below.

- **Stem cells:** Stem cell factor (also called c-KIT ligand), IL-6 and FLT3-ligand.
- **CMP Progenitor cells:**
  - Multipotent committed progenitors
    - Granulocyte-macrophage colony-stimulating factor (GM-CSF)
    - Thrombopoietin
    - IL-3, IL-5, IL-6 and IL-11
  - Committed progenitors
    - Erythropoietin
    - Granulocyte colony-stimulating factor (G-CSF)
    - Thrombopoietin
- **Lymphopoiesis** is regulated by several interleukins (most important being IL-1,2,4,5,6,7,9).

The characteristic morphologic appearances of various cells of hematopoiesis are discussed in Chapter 39.

SUMMARY

- Hematopoiesis is the continuous, regulated process of production of all blood cells.
- Organs and tissues involved in hematopoiesis are known as hematopoietic organs and are divided into myeloid and lymphoid tissue.
- The hematopoietic system is a hierarchy of cells and consists of HSC, multipotent and unipotent progenitors, morphologically identifiable precursors (blast cells) and mature cells.
- Bone marrow is the main site of hematopoiesis after birth and throughout life.
- Under abnormal conditions blood cells may be produced outside the marrow and such production is known as extramedullary hematopoiesis.

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SELF-ASSESSMENT EXERCISES

I. Short Notes

1. Extramedullary hematopoiesis
2. Hematopoietic stem cell (HSC)
3. Different sites of hematopoiesis during various phases of life
4. Functions of formed elements of blood

II. Multiple Choice Questions

1. **The hematopoietic cell which is NOT derived from the CFU-GEMM is**
   A. RBC
   B. Eosinophil
   C. Lymphocyte
   D. Platelet
2. **The cell which is most sensitive to the action of erythropoietin is**
   A. CFU-E
   B. Normoblast
   C. CFU-GEMM
   D. Reticulocyte
3. **In adults, erythropoiesis occurs mainly in**
   A. Small bones of hand and feet
   B. Flat and long bones
   C. Spleen and lung
   D. Kidneys and adrenals
4. **The cell which has the capacity for self-renewal and pluripotent differentiation is**
   A. Hematopoietic stem cell
   B. Progenitor cell
   C. Precursor cell
   D. Mature cell
5. **The transport of oxygen and carbon dioxide between the lungs and body tissues is carried out by**
   A. Red blood cells
   B. Neutrophils
   C. Basophils
   D. Platelets
6. **The commonest sites of extramedullary hematopoiesis in adult are**
   A. Liver and spleen
   B. Kidney and thymus
   C. Liver and yolk sac
   D. Lymph node and thymus
7. **In the adult, the extramedullary hematopoiesis results in**
   A. Splenomegaly
   B. Hyposplenism
   C. Atrophy of liver
   D. Atrophy of lymphnodes

ANSWERS

1. C **2.** A **3.** B **4.** A **5.** A **6.** A **7.** A

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CHAPTER 2 Classification of Anemia

CHAPTER OUTLINE

- Red cell
- Anemia

RED CELL

Mature red cell is a circular, biconcave disc containing pigmented protein called hemoglobin and is bound by the cell membrane. On a peripheral blood smear, they appear as pale **red cells with central one-third pallor** (where the upper and lower membrane surfaces closely meet). The **advantages of** the characteristic **morphology** of the RBCs are:

- The **young,** healthy red cells are **quite flexible** and **highly deformable** so that they can easily pass through extremely narrow capillary beds and splenic sinusoids. They rapidly regain their normal shape after exiting from the capillary bed.
- **Provides greater surface area** compared to volume which allows considerable alterations in the cell volume. Thus they can resist hemolysis to certain extent.

Aged RBCs become more **rigid and less deformable** and are removed from the circulation in the spleen.

Normal Parameters of Red Cell (Table 2.1)

- **Size:** The size of normal RBCs ranges from 6.7 to 7.7 μm in diameter.
- **Life span:** The average life span of normal RBC is 110-120 days.

Table 2.1: Normal range for red cell and other parameters

	At birth	*Men*	*Women*
Red cell count	5 to 7 million/cu mm ($5\text{-}7 \times 10^{12}$/L)	4.5 to 5.5 million/cu mm ($4.5\text{-}5.5 \times 10^{12}$/L)	3.8 to 4.8 million/cu mm ($3.8\text{-}4.8 \times 10^{12}$/L)
Hemoglobin (Hb)	14-22 gm/dL	13-17 gm/dL	12-15 gm/dL
Hematocrit (Hct) or packed cell volume (PCV)	45-70%	40-50%	36-46%
Reticulocyte count	1.2-4.0%	0.5-2.5%	0.5-2.5%
Red cell indices			
MCV	100-120 fL	82-100 fL	82-100 fL
MCH	31-37 pg	27-32 pg	27-32 pg
MCHC	30-36 gm/dL	31-35 gm/dL	31-35 gm/dL
RDW	13-18%	11.5-14.0%	11.5-14.0%

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- **Red cell indices** (Table 2.1 and for details refer Chapter 44)
  - **Mean cell (corpuscular) volume (MCV):** It is the average volume of a red cell and expressed in femtoliters (fL).
  - **Mean cell (corpuscular) hemoglobin (MCH):** It represents the average content (mass) of hemoglobin per red cell and expressed in picograms.
  - **Mean cell (corpuscular) hemoglobin concentration (MCHC)**: It indicates the average concentration of hemoglobin in a given volume of packed red cells and expressed in grams per deciliter.
  - **Red cell distribution width (RDW):** RDW is a quantitative measure of anisocytosis and is expressed as percentage.

ANEMIA

Definition of Anemia

- Anemia is defined as the **decrease** below normal of the **hemoglobin concentration, erythrocyte count or hematocrit**.
- It can also be defined as a **reduction of the total circulating red cell mass** below normal limits.
- **Functionally,** it is defined as the **decrease in the oxygen-carrying capacity** of the blood, which leads to tissue hypoxia.

Anemia is not a disease but it is the expression of underlying disease and from the treatment point of view, it is necessary to identify the cause of anemia.

Anemia may be **absolute**, when there is decreased red blood cell (RBC) mass, or **relative**, when associated with a higher plasma volume. Relative anemia may occur in the third trimester of pregnancy due to hemodilution (spurious anemia). But the term anemia is conventionally used for absolute anemia.

Classification of Anemia

There are several classifications of anemia. Two commonly used are:

1. **Morphological classification** (Table 2.2 and Fig. 2.1): It is based on: (1) **red cell size** (normocytic, microcytic, or macrocytic), and (2) **degree of hemoglobinization,** reflected by the color of red cells (normochromic or hypochromic).
   - **Microcytic hypochromic anemia** is characterized by red blood cells smaller than normal and the central pallor more than normal 1/3. It shows reduced MCV (< 80 fL) and reduced MCHC (30 gm/dL). It is usually due to decreased hemoglobin synthesis (e.g. iron deficiency anemia and thalassemia).
   - **Normocytic normochromic anemia** is characterized by normal red cell size as well as central pallor. It shows a normal MCV (82-100 fL) and normal MCHC (33-36 gm/dL). This type may be seen during acute blood loss.

Table 2.2: Morphological classification of anemia

- Microcytic hypochromic anemia
- Normocytic normochromic anemia
- Macrocytic normochromic anemia

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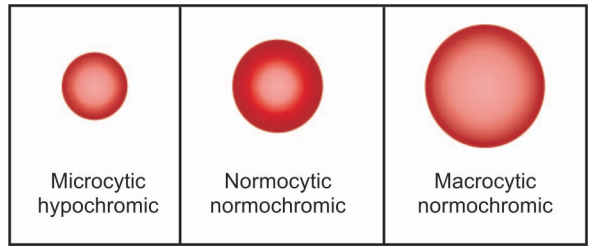


Fig. 2.1: Morphological classification of anemia

- **Macrocytic normochromic anemia** is characterized by red cells larger than normal. They have increased MCV (>100 fL) and normal MCHC and are due to defective maturation of erythroid precursors in the bone marrow. This type of anemia is commonly found with deficiency of vitamin B_{12} and folic acid.

2. **Etiological classification**: Based on the cause and the underlying mechanisms of production of anemia (Table 2.3).

Table 2.3: Etiological classification (according to underlying mechanism) of anemia

A. **Anemias due to impaired red cell production**
- Nutritional deficiencies
  - Deficiencies affecting hemoglobin synthesis: Iron deficiency
  - Deficiencies affecting DNA synthesis: Vitamin B_{12} and folic acid deficiencies
  - Vitamin C deficiency
- Immune-mediated injury to progenitors
  - Aplastic anemia
  - Pure red cell aplasia
- Primary hematopoietic neoplasms
  - Acute leukemia
  - Myelodysplastic syndromes
  - Myeloproliferative neoplasms
- Miscellaneous
  - Anemia of chronic disorders
  - Marrow suppression due to drugs

B. **Hemolytic anemias due to increased red cell destruction**
- Intracorpuscular defect
  - Hereditary hemolytic anemias
    - Red cell enzyme deficiencies
      - G6PD deficiency
      - PK deficiency
    - Red cell membrane disorders
      - Hereditary spherocytosis
      - Hereditary elliptocytosis
    - Hemoglobin abnormalities
      - Deficient globin synthesis
        Thalassemia syndrome
      - Structurally abnormal globins (hemoglobinopathies)
        Sickle cell disease
  - Acquired genetic defects: Paroxysmal nocturnal hemoglobinuria
- Extracorpuscular defect
  - Immunohemolytic anemia
  - Fragmentation syndromes
  - Hypersplenism

C. **Anemia due to blood loss**
- Acute blood loss: Trauma
- Chronic blood loss: Bleeding from GI or genitourinary tract

G6PD = Glucose-6-phosphate dehydrogenase; PK = Pyruvate Kinase; GI = Gastrointestinal

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Clinical Features of Anemia

Irrespective of the cause, anemia when severe, presents with certain clinical features. The symptoms depend on four main factors:

- **Speed of onset of anemia:** Rapidly progressive anemia causes more symptoms than that of gradual onset. This is because there is less time for adaptation in the cardiovascular system.
- **Severity of anemia:** Mild anemia produces no symptoms compared to significant symptoms in severe anemia.
- **Age of the patient:** Young patients can tolerate anemia better than elderly.
- **Underlying illness:** The disease which caused the anemia.

In anemia, the lowered oxygen content of the circulating blood leads to tissue hypoxia. General clinical features are either due to tissue hypoxia or compensatory mechanisms (Fig. 2.2).

1. **Due to tissue hypoxia:**
   - *Nonspecific symptoms:* Weakness, malaise and easy fatigability due to hypoxia of muscles.
   - *Dyspnea on mild exertion:* It is due to the lowered oxygen content of the circulating blood.
   - *Pallor:* Patients appear pale due to deficiency of red colored hemoglobin which is better appreciated in the conjunctiva, mucous membrane of tongue and nail beds. Pallor associated with icterus is suggestive of a hemolytic anemia.
   - *CNS:* Patients of severe anemia may complain of headache, vertigo, tinnitus and lack of concentration.

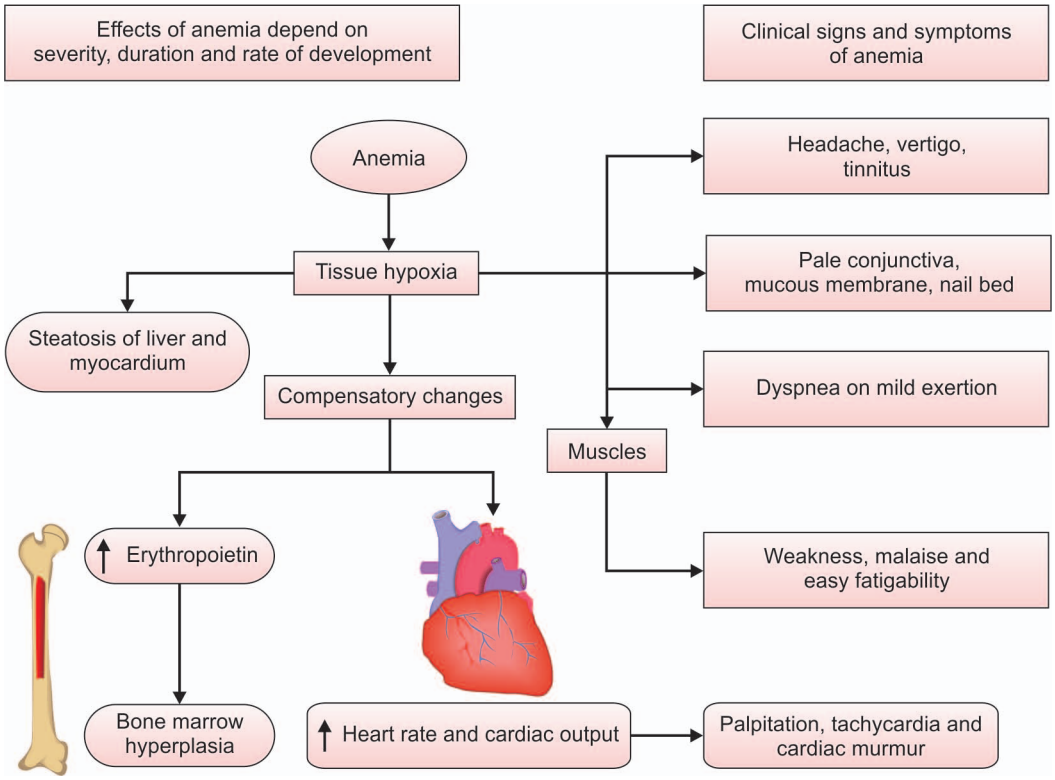


Fig. 2.2: Clinical features and effects of anemia

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2. **Due to compensatory mechanisms:**
   - *Cardiac features:* Dyspnea on mild exertion, palpitation, tachycardia and cardiac murmur occur due to compensatory mechanisms. The resulting increase in the cardiac output may cause congestive cardiac failure.

SUMMARY

- Anemia is defined as the decrease below normal of the hemoglobin concentration, erythrocyte count or hematocrit.
- Anemias are classified morphologically into normocytic, microcytic and macrocytic depending on the size of the RBCs and hypochromic or normochromic depending on the hemoglobin content.
- Anemia is classified etiologically as due to impaired production, increased destruction and blood loss.
- The symptoms of anemia are either due to the hypoxia or the compensatory mechanism.

SELF-ASSESSMENT EXERCISES

I. Short Notes

1. Red cell indices
2. Define and classify anemia.
3. Mention the morphological and etiological classification of anemia

II. Multiple Choice Questions

1. **The highest reference ranges for hemoglobin, hematocrit, and RBC count is usually found**
   A. At birth
   B. In both sexes between 1 and 5 years of age
   C. In boys older than 12 years of age
   D. In girls older than 17 years of age
2. **The following is NOT a feature of microcytic hypochromic anemia**
   A. Red blood cells smaller than normal
   B. Central pallor less than normal 1/3
   C. Reduced MCV and MCHC
   D. It is due to decreased hemoglobin synthesis

ANSWERS

1. A **2.** B

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CHAPTER 3 Iron Deficiency Anemia

CHAPTER OUTLINE

- ❖ Iron Metabolism
- ❖ Iron Deficiency Anemia

Iron is required for synthesis of normal heme of hemoglobin. Its deficiency leads to decreased erythropoiesis and anemia. Iron deficiency anemia (IDA) is the most common nutritional disorder in the world.

IRON METABOLISM

Distribution of Iron

Iron is an essential metal present in the human body. The total body iron content is divided into functional and storage compartments (Table 3.1). Iron in the body is extensively recycled between the functional and storage pools.

Table 3.1: Iron distribution in healthy young adults (in mg)

Pool	*Men*	*Women*
Total	3450	2450
Functional		
• Hemoglobin	2000-2500	1750
• Myoglobin and enzymes	350-400	300
Storage		
• Ferritin, hemosiderin	500-1000	400

1. **Functional:** Approximately 80% of the functional iron is present in hemoglobin. The remaining functional iron is found in myoglobin and iron-containing enzymes (catalase, cytochromes and peroxidases).
2. **Storage**: The storage pool contains 15-20% of total body iron. Free iron is highly toxic, because it can result in tissue damage due to its capacity to form free radicals. Therefore, iron is bound to protein and stored in the body in two forms namely; **ferritin** and **hemosiderin**. The storage iron can be readily mobilized whenever there is increase in the requirements of iron, as may occur after blood loss. Two third of the iron is stored as ferritin and one third as hemosiderin.
   - **Ferritin** is a protein-iron complex (apoferritin + iron) found in all tissues but particularly in liver, spleen, bone marrow and skeletal muscles. Very small amounts of ferritin

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circulate in the serum, the value normally being 15-300 μg/L. **Serum ferritin levels reflect the iron stores.** Serum ferritin level is usually below 12 μg/L in iron deficiency and it is very high (as high as 5000 μg/L may be observed) in conditions associated with iron overload. Ferritin is water-soluble and not visible by light microscopy.

- **Hemosiderin** is an aggregate of iron and protein which is found in the reticuloendothelial cells of bone marrow, spleen and liver. Hemosiderin appears as golden yellow granules in the cytoplasm of the cells when stained with routine Hematoxylin and Eosin. When treated with **Prussian blue (Perl's) stain,** hemosiderin takes up blue-black color due to the iron reacting with potassium ferrocyanide of Perl's stain. In iron-overload, most of the iron is stored in the cells as hemosiderin.

Daily requirements: The daily requirement in adult males is 5-10 mg/day and in females 20 mg/day.

Dietary sources: The diet contains iron either in the form of **heme** contained in animal products and/or **nonheme** iron in vegetables.

Iron Absorption

- *Site of absorption:* Iron is absorbed from the **duodenum** and **proximal jejunum**. Iron balance is maintained mainly by regulating the dietary absorption of iron.
- *Factors influencing absorption* (Table 3.2)

Table 3.2: Factors affecting iron absorption

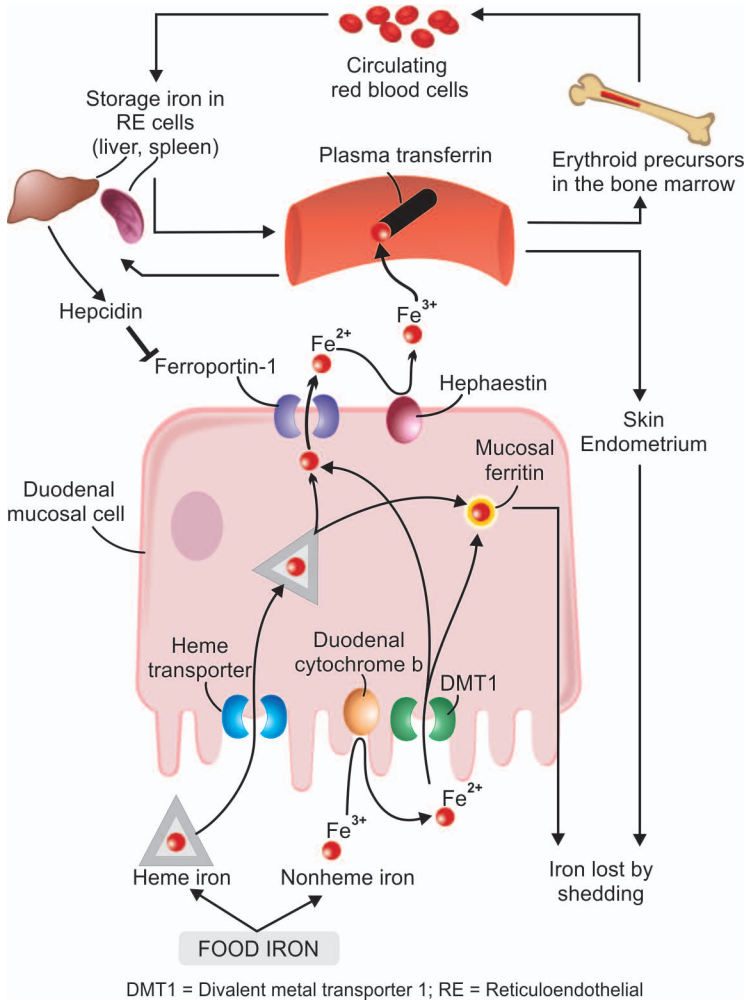
Factors that favor absorption	*Factors that inhibit absorption*
Heme iron	Inorganic iron
Ferrous form	Ferric form
Acids (ascorbic acid, citric acid, hydrochloric acid of the stomach)	Phytates of cereals
Solubilizing agents (sugars in the diet, amino acid)	Tannates (as in tea), carbonates, oxalates and phosphates of diet
Reducing substances in the diet convert ferric iron to ferrous form which is readily absorbed	Drugs

Molecular Aspect of Iron Absorption (Fig. 3.1)

- Nonheme iron:
  - The absorption of heme iron is better than nonheme. Iron in nonheme form is mostly in the Fe^{3+} (ferric) state and is reduced to Fe^{2+} (ferrous) iron by ferrireductases, such as duodenal cytochrome b.
  - Fe^{2+} iron is then transported across the brush border of the cytoplasmic membrane of the enterocyte (duodenal or jejunal epithelial cells) by **divalent metal transporter 1 (DMT1).**
- Heme iron is moved across the brush border of the cytoplasmic membrane of the enterocyte through heme transporters into the cytoplasm.

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DMT1 = Divalent metal transporter 1; RE = Reticuloendothelial

Fig. 3.1: Metabolism and molecular aspects of iron absorption

Iron (heme or nonheme) that enters the cytoplasm of the enterocyte may be either transported to the blood or stored as mucosal iron. Fe^{2+} iron destined for transport from cytoplasm to the circulation is carried out by **ferroportin-1**. This process is coupled by the iron oxidase like hephaestin (which is expressed predominantly in the enterocyte) which oxidizes Fe^{2+} iron to Fe^{3+} form.

Note: Both DMT1 and ferroportin are also involved in iron transport in other tissues and are distributed widely in the body.

Transport of Iron

Iron is transported in plasma by the iron transport protein **transferrin**, which is synthesized in the liver. The major function of plasma transferrin is to deliver iron to erythroid precursors

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for the synthesis of hemoglobin. In normal individuals, transferrin is about 33% saturated with iron, with an average serum iron levels of 120 µg/dL in men and 100 µg/dL in women. Thus, the total iron-binding capacity of serum is in the range of 310 to 340 µg/dL.

Iron Excretion

Iron metabolism is unique as it is very efficiently utilized and reutilized by the body. There is no physiological regulated mechanism for iron excretion and 1 to 2 mg per day is lost by shedding of epithelial cells of GI tract, skin epithelial cells (by sweat) and renal tubules.

Regulation of Iron Balance

Iron is essential for cellular metabolism; at the same time excess of it is highly toxic. Therefore, the total body iron stores must be properly regulated. Iron balance is mainly achieved by regulating the absorption of iron in the diet. As body stores decreases, the absorption of iron rises and vice versa. **Hepcidin** synthesized in the liver is the **central regulator of iron homeostasis** and it **controls absorption and storage**. Hepcidin inhibits iron transfer from the enterocyte to plasma by binding to ferroportin. When hepcidin levels rise, iron gets stored within enterocytes forming mucosal ferritin and is shed with the cells (Fig. 3.1).

IRON DEFICIENCY ANEMIA

Etiology of Iron Deficiency Anemia (Table 3.3)

Table 3.3: Causes of iron deficiency anemia

1. **Dietary deficiency/lack**
   - Milk fed infants because of low iron content in breast milk
   - Elderly with improper diet and poor dentition
   - Low socioeconomical sections of the society
   - Vegetarians (contains poorly absorbable inorganic iron)
2. **Impaired absorption**
   - Total/partial gastrectomy impairs iron absorption by decreasing hydrochloric acid and transit time through the duodenum
   - Intestinal absorption is impaired in sprue, other causes of intestinal steatorrhea and chronic diarrhea
   - Specific items in the diet, like phytates of cereals, tannates, carbonates, oxalates, phosphates and drugs can impair iron absorption
3. **Increased demand/requirement**
   - Growing infants, children and adolescents
   - Pregnancy and lactation
4. **Chronic blood loss**
   Due to bleeding from the
   - Gastrointestinal tract (e.g. peptic ulcers, gastric carcinoma, colonic carcinoma, hemorrhoids, hookworm infestation or nonsteroidal anti-inflammatory drugs)
   - Urinary tract (e.g. renal or bladder tumors)
   - Genital tract (e.g. menorrhagia, uterine cancer)
   - Respiratory tract

In adult men and postmenopausal women, deficiency must be attributed to gastrointestinal blood loss unless otherwise proven.

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Pathogenesis of Iron Deficiency Anemia

Iron deficiency anemia develops progressively through three stages (Fig. 3.2).

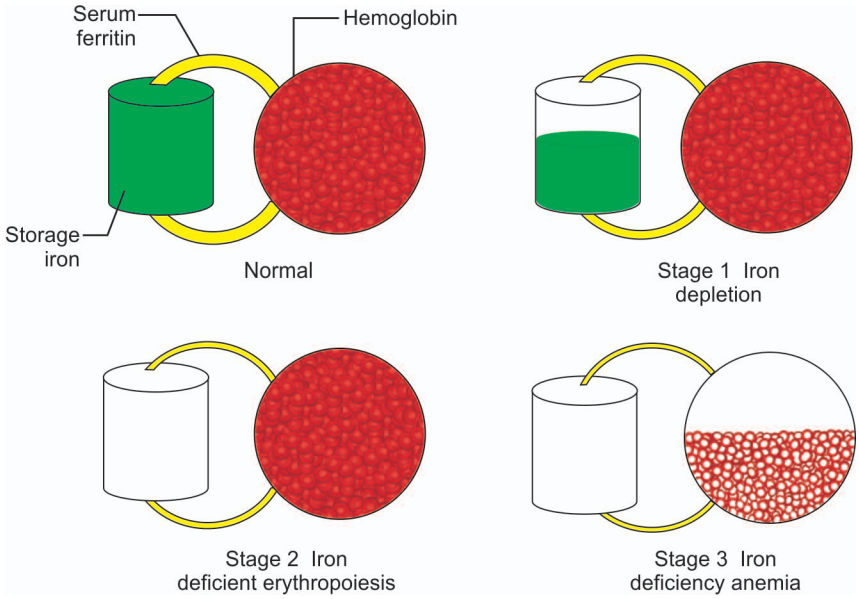


Fig. 3.2: Stages of iron deficiency anemia

Stage 1 (Iron depletion): In the initial phase, iron may be adequate to maintain normal levels of hemoglobin and **only serum ferritin** levels are **decreased.**

Stage 2 (Iron deficient erythropoiesis): Progressive depletion of iron reserves first **lowers serum iron and transferrin saturation levels** without producing anemia (Hb, MCV and MCH within normal range). Bone marrow, at this stage, shows iron deficient erythropoiesis.

Stage 3 (Iron deficiency anemia): Anemia only appears when depleted **iron stores** are accompanied by low serum iron, serum ferritin and transferrin saturation. As the serum iron level falls and transferrin saturation decreases below the critical value of <15%, the hemoglobin production is impaired. Morphologically, there is **first reduction in the size (microcytic) and later increase in the central pallor (hypochromia) of red blood cells.** Normally, increasing cytoplasmic hemoglobin concentration acts as an inhibitor of normoblast division. In iron deficiency, the failure of hemoglobin synthesis allows extra mitoses (cell division) to occur during erythropoiesis, with the production of small erythrocytes (microcyte).

When anemia is very severe, misshapen red blood cells (poikilocytes) are formed resulting in ineffective erythropoiesis. Many other iron proteins/enzymes like cytochrome c, cytochrome oxidase and myoglobin are reduced. Diminution of tissue enzymes is responsible for characteristic epithelial changes found in iron deficiency anemia.

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Clinical Features

The clinical manifestations of the anemia are **nonspecific.** The presenting signs and symptoms are frequently related to both anemia and the underlying cause of the anemia (gastrointestinal or gynecologic disease, malnutrition, pregnancy and malabsorption).

- **Onset:** It is **insidious** and symptoms appear as anemia progresses. Patients become symptomatic as hemoglobin falls below 7 gm/dL.
- **Nonspecific symptoms:** Most of the patients complain of nonspecific symptoms like fatigue, palpitations, breathlessness, weakness and irritability. These symptoms worsen as the severity of anemia increases.
- **Pharyngeal/esophageal webs:** Folds (webs) of mucosa are formed at the junction of the hypopharynx (post-cricoid region) with esophagus. These webs cause dysphagia (difficulty in swallowing) mainly to solid foods. Triad of major findings in the **Patterson-Kelly or Plummer-Vinson syndrome** are:
  - Microcytic hypochromic anemia
  - Atrophic glossitis
  - Esophageal webs in middle aged women.

  These mucosal abnormalities are premalignant and occasionally carcinoma develops at the site.
- **Congestive heart failure:** With severe anemia, patient develops a hyperdynamic circulation and may present with congestive heart failure.
- **Central nervous system:** Appearance of pica, which is an unusual craving for certain substances with no nutritional value like clay or chalk. Craving for ice (pagophagia) is believed to be the most specific to iron deficiency.

Physical Findings

- **Angular stomatitis and glossitis:** Atrophy of papillae gives a red, smooth, waxy appearance to tongue surface. Fissures and ulcerations at the angles of mouth (angular stomatitis) may develop.
- **Chronic atrophic gastritis:** Atrophic changes in the gastric mucosa lead to **chronic atrophic gastritis** with hypochlorhydria.
- **Koilonychia**: Fingernails become thin, flattened (platynychia), ridged, brittle and spoon-shaped (koilonychia). This is due to depletion of essential iron-containing enzymes in cells.

Laboratory Findings

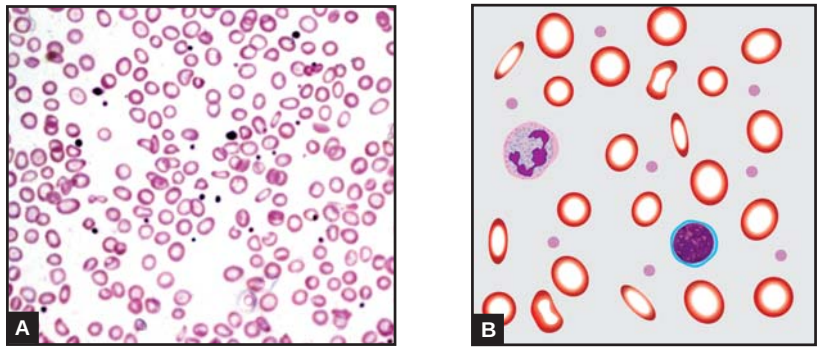
Peripheral Blood

- **Hemoglobin:** Hemoglobin levels are **decreased** and usually in the range of 6 to 10 gm/dL at the time of diagnosis. Severe anemia with hemoglobin as low as 3 gm/dL is also encountered.
- **Hematocrit:** It is **reduced** (13-30%) and sometimes it may be much lower because of microcytic red cells.

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- **Red cell indices:**
  - **Mean corpuscular volume(MCV) reduced** (< 80 fL)
  - **Mean corpuscular hemoglobin(MCH) reduced** (< 25 pg)
  - **Mean corpuscular hemoglobin concentration (MCHC) reduced** (< 27 gm/dL)
  - **Red cell distribution width (RDW) increased.** It is the earliest sign of iron deficient erythropoiesis.
- **Peripheral smear:**
  - **RBCs:**
    - Red blood cells are **microcytic** (small) and **hypochromic** (pale). The red cells are smaller in diameter than the nucleus of a typical small lymphocyte, and are called microcytes (Figs 3.3A and B). Normal well-hemoglobinized red cells have a zone of central pallor measuring about one third of the cell diameter. When the central pallor is more than normal 1/3 it is known as hypochromia. In severe anemias, the central pallor becomes 2/3 to 3/4 of the red cell and only peripheral rim of hemoglobin is seen (ring/pessary cells).
    - There is moderate degree of **anisocytosis** and **poikilocytosis.** Poikilocytosis in the form of small, elongated red cells (pencil/cigar shaped cells) is also characteristic.
  - **WBCs:** Total leukocyte count and differential count are usually normal.
  - **Platelets:** The count may be normal but is often raised, especially if chronic bleeding is present.



Figs 3.3A and B: Peripheral blood smear showing microcytic hypochromic red blood cells

Occasionally, a mixture of microcytic hypochromic and macrocytic red blood cells is seen. This is termed as dimorphic picture (see Fig. 4.6) and occurs in mixed deficiency of iron and folic acid or vitamin B_{12}. The MCV and MCH may be misleadingly normal.

- **Reticulocyte count:** It is low for the degree of anemia.

Bone Marrow

- **Cellularity:** Bone marrow is moderately **hypercellular**.
- **M:E ratio:** Varies from 2:1 to 1:2 (normal 2:1 to 4:1)
- **Erythropoiesis:** Shows **hyperplasia** but is less as compared to the degree of anemia.

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- **Micronormoblastic maturation:** Erythroid precursors are smaller than normoblasts and are called micronormoblasts. The late micronormoblasts show persistent basophilia with frayed cytoplasmic borders due to lack of complete hemoglobinization.

- **Myelopoiesis:** Normal.
- **Megakaryopoiesis:** Normal.
- **Absence of bone marrow iron:** Decreased or absent bone marrow iron is the "gold standard" test for iron deficiency, which is revealed by negative Prussian blue reaction.

Serum Iron Profile

The various serum measurements of iron status in iron deficiency anemia (Table 3.4) can be classified as tests that detect iron stores, tissue iron supply or functional iron.

Table 3.4: Serum iron profile in IDA

	Normal range	*Value in IDA*	*Observation*
Serum ferritin	15-300 µg/L	<15µg/L	Decreased
Serum iron	50-150 µg/dL	10-15 µg/dL	Decreased
Serum transferrin saturation	30-40%	<15%	Decreased
Total plasma iron-binding capacity (TIBC)	310-340 µg/dL	350-450 µg/dL	Increased
Serum transferrin receptor (TFR)	0.57-2.8 µg/L	3.5-7.1 µg/L	Increased
Red cell protoporphyrin	30-50 µg/dL	>200 µg/dL	Increased

a. Iron stores:

- **Serum ferritin**
  Indicates the iron store status and **decreased** serum ferritin is the **first, most sensitive** and **specific test** for iron deficiency. The levels <15 µg/L indicate depletion of iron stores (normal levels 15-300 µg/L) and is diagnostic of iron deficiency.
  Because ferritin is an acute phase reactant protein, it is not reliable in the presence of infection, inflammation, hemolysis and neoplasia. In those situations serum ferritin may be normal or high despite tissue iron depletion.
- **Reduced iron stores** inhibit hepcidin synthesis and its serum levels are decreased.

b. Tissue iron supply

- **Serum iron: Reduced** to 10-15 µg/dL (normal 50-150 µg/dL).
- **Serum transferrin saturation:** Transferrin is the major physiological transport protein for iron in the blood, which transports iron to cells. Transferrin saturation is the proportion of available sites on transferrin for iron binding that is occupied by iron atoms and is expressed as percentage. Normally, only 33% (normal 30-40%) of transferrin is saturated. Low serum iron with increased iron-binding capacity results in a **reduction of transferrin saturation levels to below 15%.**
- **Total plasma iron-binding capacity (TIBC):** Transferrin has the capacity to bind approximately 300 µg/dL (normal 310-340 µg/dL) of iron. This value is known as total plasma iron-binding capacity. This is **increased** to 350-450 µg/dL.

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- **Serum transferrin receptor (TfR) assays: Serum transferrin receptor protein** levels are **increased** in iron deficiency anemia and helps in differentiating it from anemia of chronic disorders in which it is in the normal range of 4-8 μg/L.
- **Red cell protoporphyrin:** Reduced heme synthesis leads to **elevation of free erythrocyte protoporphyrin** to >200 μg/dL (Normal 30-50 μg/dL).

c. **Functional iron: Decreased** functional iron results in reduced Hb, MCV and MCH.

Other Causes of Microcytic Hypochromic Anemia

- **Thalassemia major:** Presents with features of hemolytic anemia and hepatosplenomegaly. The iron stores are increased.
- **Anemia of chronic disorders:** Clinical features of underlying chronic disorders are present and iron stores are either normal or increased.
- **Other causes:** These include alcohol, lead poisoning and drugs.

SUMMARY

- Iron is an important nutrient required for the synthesis of hemoglobin.
- Iron is present in heme or nonheme form in the diet. Heme form is absorbed better than nonheme form and absorption occurs in the duodenum and jejunum via DMT1 transporter.
- Hepcidin is the central regulator of iron homeostasis and it controls its absorption and storage.
- Iron is stored as ferritin and hemosiderin, the latter stains blue with Prussian blue reaction.
- Iron deficiency anemia is the most common nutritional disorder in the world.
- The causes of iron deficiency anemias are: dietary deficiency, impaired iron absorption, increased demand/requirement or chronic blood loss.
- Lab findings in iron deficiency anemia



Low hemoglobin levels
Low hematocrit
Low serum iron levels
Low serum ferritin levels
Low RBC count with microcytic and hypochromic cells
Decreased MCV/MCH in severe anemia
Decreased bone marrow iron stores
Increased total plasma iron-binding capacity (TIBC).

SELF-ASSESSMENT EXERCISES

I. Essay

Define and classify anemia. Discuss the laboratory investigations in iron deficiency anemia.

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II. Short Notes

1. Write a note on peripheral blood picture and bone marrow changes in iron deficiency anemia
2. Laboratory findings in iron deficiency anemia
3. List the causes and laboratory diagnosis of microcytic hypochromic anemia

III. Multiple Choice Questions

1. Iron deficiency anemia may develop in all the following conditions EXCEPT
A. Billroth II operation
B. Capillary hemangioma
C. Carcinoma colon
D. Hookworm infestation

2. Earliest response during treatment of iron deficiency anemia is increase in
A. Hemoglobin level
B. Packed cell volume
C. Reticulocyte count
D. Percentage of neutrophils

3. The substance in the diet which favors iron absorption is
A. Ascorbic acid
B. Oxalate
C. Phytate
D. Tannate

4. The substance in the diet which inhibits iron absorption is
A. Vitamin C
B. Citric acid
C. Sugar
D. Tannate

5. The following is increased in iron deficiency anemia
A. Serum iron
B. Serum ferritin
C. Total iron binding capacity
D. Ceruloplasmin

6. The following laboratory findings which is NOT a feature of iron deficiency anemia
A. Decreased bone marrow iron
B. Decreased red cell protoporphyrin
C. Microcytic hypochromic anemia
D. Increased total iron binding capacity

7. The blood index which most accurately reflects iron deficiency is
A. MCV
B. MCH
C. MCHC
D. PCV

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8. The following is the most accurate estimate of iron stores in the body
A. Serum iron
B. Serum ferritin
C. Prussian blue reaction
D. Total iron binding capacity

9. The following is NOT a feature of Plummer-Vinson syndrome
A. Decreased total iron binding capacity
B. Microcytic hypochromic anemia
C. Atrophic glossitis
D. Esophageal web

ANSWERS

1. B **2.** C **3.** A **4.** D **5.** C
6. B **7.** A **8.** B **9.** A

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CHAPTER 4 Megaloblastic Anemia

CHAPTER OUTLINE

- Introduction
- Definition
- Metabolism of Vitamin B_{12} and Folic Acid
- Etiology of Megaloblastic Anemia
- Laboratory Findings of Megaloblastic Anemia
- Anemias of Vitamin B_{12} Deficiency
- Anemia of Folate Deficiency
- Nonmegaloblastic Causes of Macrocytic Anemias

INTRODUCTION

Macrocytic anemias are characterized by large red cells with a diameter of more than 9μ and mean corpuscular volume of more than 100 fl. The causes of macrocytic anemias may be broadly divided into megaloblastic and non-megaloblastic depending on the appearance of developing red cell precursors in the bone marrow.

Megaloblastic anemias are characterized by the presence of abnormal red cell precursors in the bone marrow known as megaloblasts. The megaloblasts differ from their normal counterpart normoblasts in several aspects (Table 4.1).

Table 4.1: Features of megaloblast

Characteristics	*Megaloblast*
Cell size	Larger than normoblast
Nuclear chromatin	More open-sieve like
Dissociation of cytoplasmic and nuclear maturation	Nuclear maturation lags behind cytoplasmic maturation
Maturation	Marrow shows increased proportion of more primitive erythroid cells

Megaloblastic anemias are common among anemias due to impaired red cell production, being second in incidence to iron deficiency and anemia of chronic disorders.

DEFINITION

Megaloblastic anemias are diverse group of anemias characterized by **impaired DNA synthesis** and **distinct morphologic changes** in hematopoietic cells, i.e. maturation of nucleus being delayed in relation to that of cytoplasm.

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- As the name implies, there is anemia with distinct morphologically abnormally large erythroid precursors in the **bone marrow** known as **megaloblasts**.
- Consequently, the red cells in the peripheral blood are also larger than normal and are termed as **macrocytes**.

Cause of Megaloblastic Change and Macrocytic Red Cells

- Megaloblastic anemia is commonly due to deficiency of **vitamin B_{12} (cyanocobalamin) or folic acid**, which are coenzymes required for the synthesis of one of the four nucleotide bases found in DNA namely thymidine.
- Deficiency of vitamin B_{12} or folic acid causes **defects in the DNA synthesis** and nuclear maturation with delay or block in cell division.
- Synthesis of RNA and protein is normal resulting in normal cytoplasmic maturation.
- Thus, the **nuclear maturation lags behind the cytoplasmic maturation** and hemoglobinization of cytoplasm continues for a long time. This nuclear to cytoplasmic asynchrony results in formation of large nucleated erythrocyte precursors named as **megaloblasts.** The megaloblasts have an open, stippled, lacy chromatin pattern.
- Since megaloblast precursors do not mature enough to be released into the blood, they undergo intramedullary destruction. There is also mild hemolysis of red cells in the peripheral blood.
- Erythropoietic cells show reduced number of mitoses which results in the production of enlarged red cells namely **macrocytes.**
- All rapidly dividing cells of the body (including skin, GI tract, bone marrow) exhibit megaloblastic changes and anemia is only a manifestation of a more generalized defect in DNA synthesis.

METABOLISM OF VITAMIN B_{12} AND FOLIC ACID

Metabolism of vitamin B_{12} and folic acid are closely related and both are essential for normal DNA synthesis and nuclear maturation. Other disorders may be associated with macrocytosis but megaloblastic hematopoiesis is most commonly due to deficiency of vitamin B_{12} or folic acid.

Vitamin B_{12} Metabolism

Human beings are totally dependent on animal products in the diet for vitamin B_{12} requirement. Vitamin B_{12} is not present in food from vegetable sources. Therefore, strict vegetarians do not get an adequate quantity of vitamin B_{12}. A balanced diet (not rigid vegetarian!) contains significantly large amounts of vitamin B_{12} which accumulates in the body (liver) and is enough for several years. Due to this adequate storage, if there is any dietary deficiency or malabsorption of vitamin B_{12}, its clinical manifestations appear only after about 2 to 4 years. Vitamin B_{12} is a complex compound known as cobalamin and daily requirement is about 2 to 3 μg.

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Absorption, Transport and Storage (Fig. 4.1)

- Vitamin B_{12} in food is usually in coenzyme form (as deoxyadenosylcobalamin and methylcobalamin) and bound to proteins in the diet.
- In the stomach, peptic digestion at low pH is required for release of vitamin B_{12} from food protein. The released vitamin B_{12} binds with R protein, which is secreted in salivary and gastric juices.
- These vitamin B_{12}–R protein complexes leave the stomach along with unbound special protein called **intrinsic factor** (IF), which is produced by gastric (fundus and cardia) parietal (oxyntic) cells.
- As the vitamin B_{12}–R protein complexes pass into the second part of the duodenum, pancreatic proteases degrade the R protein and released vitamin B_{12} forms complexes with intrinsic factor.
- This stable IF-vitamin B_{12} complex is transported to the ileum, where it binds to intrinsic factor-specific receptors on microvilli present on the surface of ileal mucosal cells.

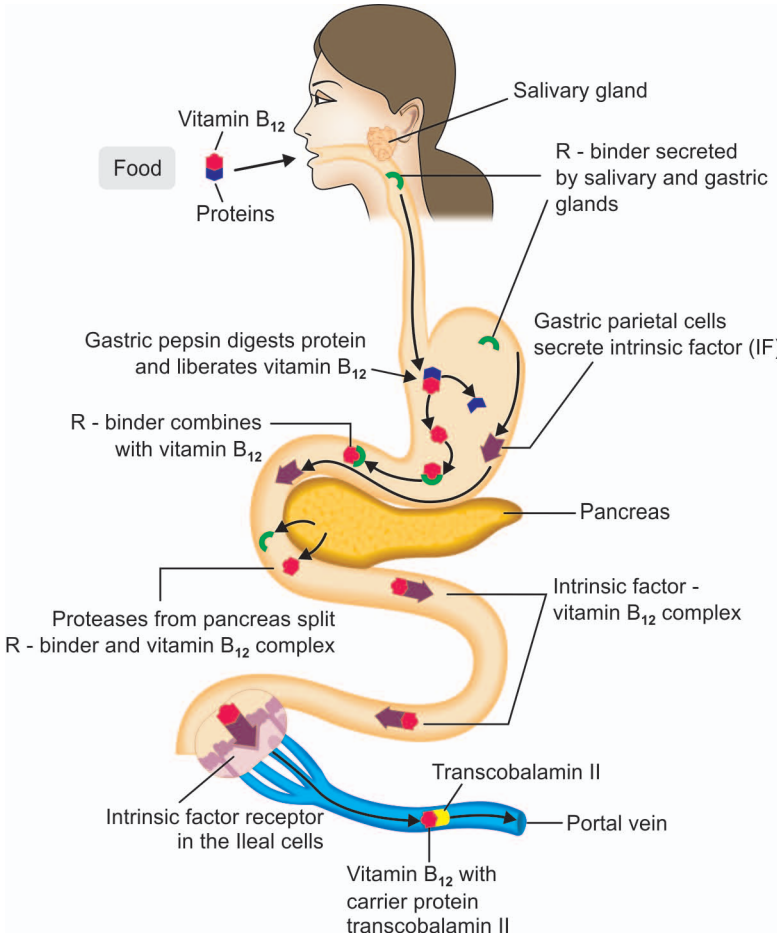


Fig. 4.1: Mechanism of vitamin B_{12} absorption

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- In the ileal epithelium, vitamin B_{12} combines with a major carrier protein, transcobalamin II, and is actively transported into the mucosal cells and then into the blood.
- Transcobalamin II-vitamin B_{12} complex delivers vitamin B_{12} to the liver and other cells of the body, particularly rapidly proliferating cells in the bone marrow and mucosal lining of the gastrointestinal tract.

Role of Vitamin B_{12}

Vitamin B_{12} is indirectly required for DNA synthesis in various metabolic steps and its deficiency impairs DNA synthesis.

- Methylcobalamin is the main form of vitamin B_{12} in plasma, and is an essential coenzyme for conversion of homocysteine to methionine and formation of tetrahydrofolate (THF) from methyl THF (Fig. 4.2). During the former reaction, vitamin B_{12} loses its methyl group and this is replaced from methyl THF, the principal form of folic acid in plasma. Tetrahydrofolate is essential for the generation of a precursor of DNA known as deoxythymidine monophosphate (dTMP).
  In vitamin B_{12} deficiency, the main cause of impaired DNA synthesis is that methyl THF is not converted into THF. Methyl THF accumulates in the cell and is known as **methyl THF trap.**
- Vitamin B_{12} is also required for conversion of methylmalonyl CoA to succinyl malonyl CoA (Fig. 4.3). Deficiency of vitamin B_{12} causes increased levels of methylmalonic acid in plasma and urine. This results in the formation of abnormal fatty acids which get incorporated into

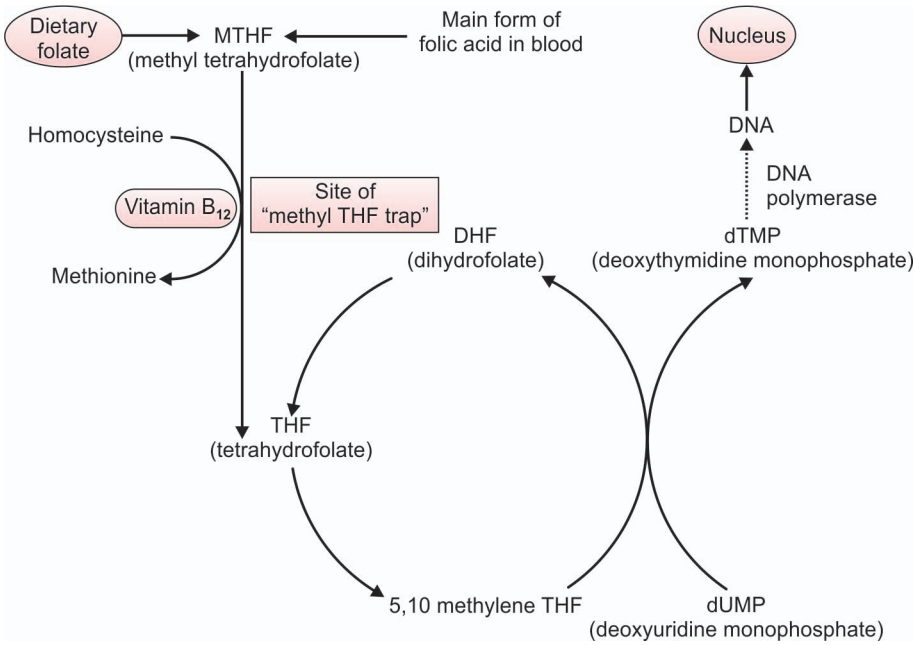


Fig. 4.2: Interrelation and role of vitamin B_{12} and folic acid in DNA synthesis

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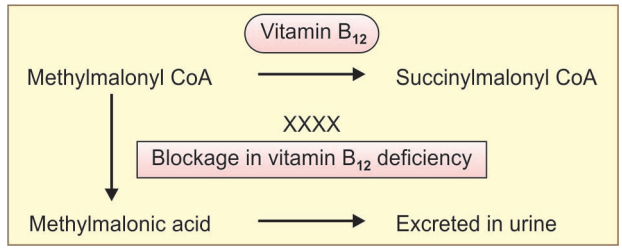


Fig. 4.3: Role of vitamin B_{12} in methylmalonyl CoA metabolism

neuronal lipids. Consequently, this predisposes to myelin breakdown and is probably responsible for neurologic complications of vitamin B_{12} deficiency.

Folic Acid Metabolism

Humans are entirely dependent on dietary sources for their folic acid requirement. The daily requirement is 50 to 200 mg. Green vegetables, yeast, legumes, fruits and animal proteins are the richest sources and most normal diets contain sufficient amounts of folic acid. The folic acid in these foods is largely in the form of polyglutamates. Polyglutamates are sensitive to heat (thermolabile); boiling, steaming or frying and cooking destroys most of the folic acid. Intestinal conjugases split the polyglutamates into monoglutamates that are readily absorbed in the proximal jejunum. During intestinal absorption, they are modified to 5 methyltetrahydrofolate, the normal transport form of folic acid.

Role of Folic Acid (FA)

The active form of folic acid is tetrahydrofolate [THF] which is the biologic "middleman" involved in metabolic processes which synthesize DNA. The various reactions in which folic acid plays a main role are:

- Synthesis of purine (required for DNA and RNA)
- Conversion of homocysteine to methionine, a reaction also requiring vitamin B_{12}
- Conversion of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP): 5,10-methylene THF polyglutamate is required for conversion of d UMP to dTMP and DNA, a rate limiting step in pyrimidine synthesis (Fig. 4.2).
- Metabolism of histidine: Histidine is metabolized to formiminoglutamic acid (FIGLU) which combines with THF to form glutamic acid (Fig. 4.4). In FA deficiency, this reaction cannot take place and therefore FIGLU accumulates and is excreted as such in urine. This is used as a test to measure folic acid deficiency.

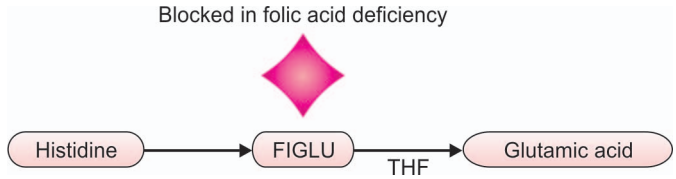


Fig. 4.4: Role of folic acid in metabolism of histidine

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ETIOLOGY OF MEGALOBLASTIC ANEMIA

Megaloblastic anemia is commonly due to deficiency of **vitamin B_{12} (cobalamin) or folic acid** (Table 4.2).

Table 4.2: Causes of megaloblastic anemia

VITAMIN B_{12} DEFICIENCY

Decreased intake

Inadequate diet, "pure vegetarians"

Impaired absorption

Deficiency of gastric acid or pepsin or intrinsic factor

- Pernicious anemia
- Post gastrectomy

Intestinal

- Loss of absorptive surface
  - Malabsorption syndromes
  - Diffuse intestinal disease (e.g. lymphoma, systemic sclerosis)
  - Ileal resection, Crohn disease
- Bacterial or parasitic competition for vitamin B_{12}
  - Bacterial overgrowth in blind loops and diverticula of bowel
  - Fish tapeworm infestation

Damage to exocrine pancreas

Increased demand

Pregnancy, hyperthyroidism, disseminated cancer

FOLIC ACID DEFICIENCY

Decreased intake

Inadequate diet: alcoholism, malnutrition

Impaired absorption

Malabsorption states: Nontropical and tropical sprue

Diffuse infiltrative diseases of the small intestine (e.g. lymphoma)

Drugs: Phenytoin and oral contraceptives

Increased loss

Hemodialysis

Increased demand

Pregnancy, infancy, disseminated cancer, markedly increased hematopoiesis

Impaired utilization

Folic acid antagonists, such as methotrexate

LABORATORY FINDINGS OF MEGALOBLASTIC ANEMIA

Certain features common to all forms of megaloblastic anemia are discussed together. Blood findings in vitamin B_{12} and/or folic acid deficiency are similar and are characterized by macrocytosis of red cells and megaloblastosis of bone marrow.

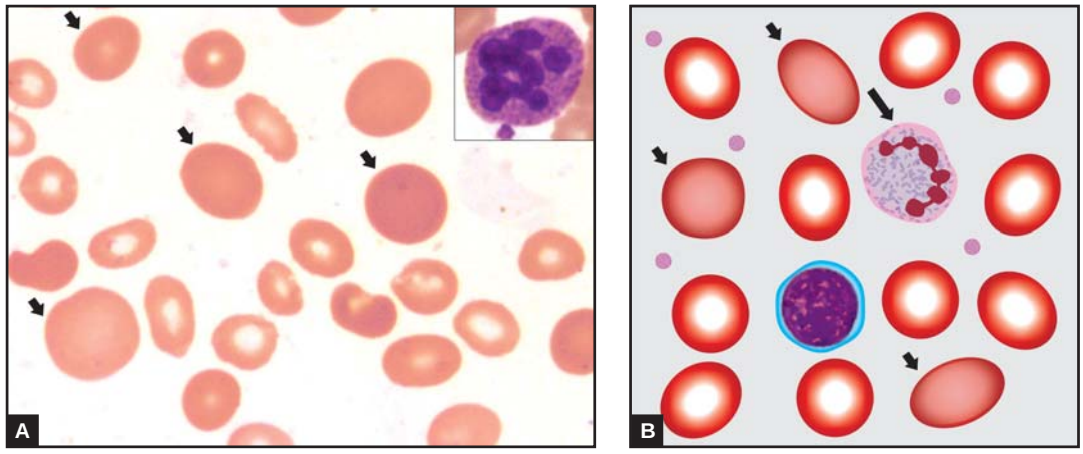
Peripheral Blood

- **Hemoglobin:** Hemoglobin levels are decreased and usually in the range of 5 to 10 gm/dL.
- **Hematocrit:** Decreased

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- **Red cell indices:**
  - **Mean cell (corpuscular) volume (MCV) above 100 fle (normal 82 to 98).**
  - Hemoglobin content in the red cell is proportionately increased and therefore **mean corpuscular hemoglobin concentration (MCHC)** remains **normal.**
  - **Mean cell (corpuscular) hemoglobin (MCH) is increased.**
- **Peripheral smear**
  - **RBCs: Red blood cells** are of variable size with majority being **macrocytic and oval (macro-ovalocytes)** and are diagnostic of megaloblastic anemia. Macrocytes are larger in diameter, thickness and volume. Because macrocytes are thicker and well-hemoglobinized, most **macrocytes lack the central pallor** of normal red cells and can even appear hyperchromic (Figs 4.5A and B). There is marked variation in the size and shape of red cells (anisopoikilocytosis).



Figs 4.5A and B: Peripheral blood smear showing macrocytes (short arrows) and hypersegmented neutrophil (inset in A and long arrow in B)

  - Evidences of dyserythropoiesis (refer page 34) may be present like:
    - Basophilic stippling (see Fig. 42.5) appear as blue black cytoplasmic inclusions and represents precipitated ribosomal RNA.
    - Cabot ring (see Fig. 42.5) which stains pink blue and represents nonhemoglobin iron.
    - Howell Jolly bodies (see Fig. 42.5) are nuclear remnants observed in few red cells.
  - **WBCs: White cells** are decreased (leukopenia) and show **hypersegmented neutrophils (with five to six or more nuclear lobes)**. Their presence is the **first** and specific morphologic **sign** of megaloblastic anemia. Such **neutrophils are** also **larger than normal (macropolymorphonuclear).**
  - **Platelets:** Decreased (thrombocytopenia) and the count varies.

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Dimorphic anemia: In cases of combined vitamin B_{12}/folic acid and iron deficiency, peripheral smear demonstrates dual population of macrocytes and hypochromic microcytes (Fig. 4.6). However, bone marrow shows normoblastic reaction with giant metamyelocytes and complete absence of marrow iron.

- **Reticulocyte count:** It is normal or low. Reticulocytosis does not occur since it is a dyserythropoietic anemia. Nucleated red cells occasionally appear in the circulating blood with severe anemia.

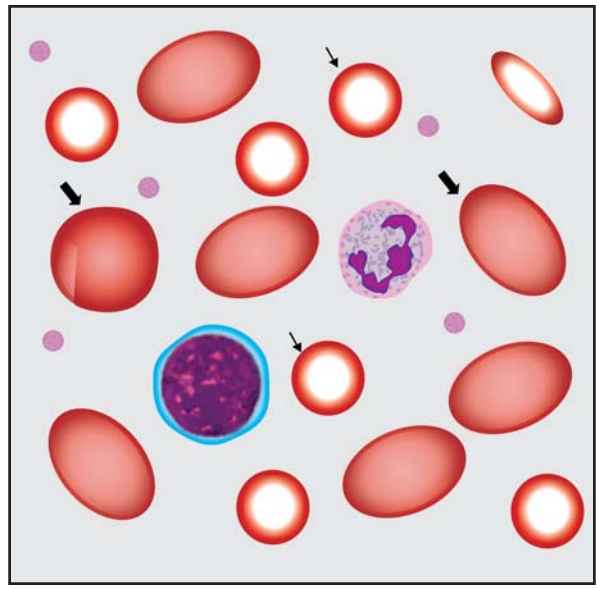


Fig. 4.6: Peripheral blood smear showing both microcytic hypochromic cells (narrow arrow) and macrocytes (dimorphic anemia)

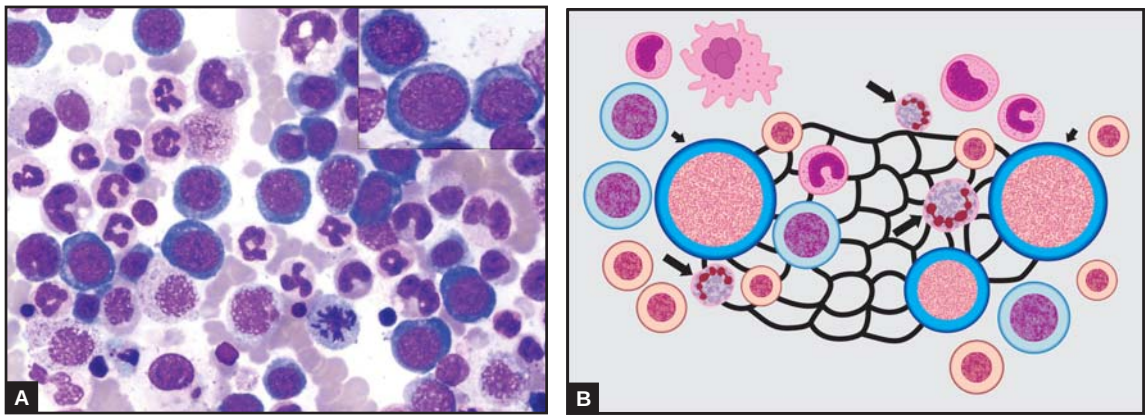
Bone Marrow

Bone marrow shows characteristic morphological features.

- **Cellularity:** Marrow is moderately to **markedly hypercellular** mainly due to proliferating erythroid precursors which may completely replace the fatty marrow.
- **M:E ratio**: Because of marked erythroid hyperplasia, M:E ratio is reversed ranging from 1:1 to 1:6 (normal 2:1 to 4:1).
- **Erythropoiesis:** It is of **megaloblastic type** (Figs 4.7A and B) in contrast to normal normoblastic type.

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Figs 4.7A and B: Bone marrow aspirate showing megaloblastic precursors (short arrows) in varying stages of maturation (inset in A shows early megaloblasts). B also shows hypersegmented neutrophils (long arrows)

- **Megaloblasts:** These are **large, abnormal counterparts of normal normoblasts** and their characteristic features are presented in Table 4.1 and Fig. 4.8. Megaloblastic change is detected in all stages of red cell development. They demonstrate **asynchrony of nuclear and cytoplasmic maturation**, nuclear chromatin failing to mature because of impaired DNA synthesis while cytoplasm gets normal hemoglobinization.
- **Ineffective erythropoiesis:** In an untreated megaloblastic anemia there is preponderance of more primitive cells. The late stage megaloblast forms die in marrow and are known as ineffective erythropoiesis (or intramedullary hemolysis).
  Note: Ineffective erythropoiesis is the term used for erythropoiesis in which there is death of developing erythroid cells at the site of production and/or production of non-viable red cells.
- **Dyserythropoiesis:** Dyserythropoiesis means abnormal erythropoiesis with bizarre bone marrow morphology and ineffective erythropoiesis. The dyserythropoiesis in the megaloblasts demonstrates the following morphological features:
  - In the cytoplasm: Howell Jolly bodies (see Fig. 42.5) and abnormal hemoglobinization
  - In the nucleus: Irregular nuclear borders due to nuclear budding, nuclei joined by bridge (internuclear bridging) and abnormal mitosis

- **Myelopoiesis**
  - Myeloid cells appear adequate in number, but patients with severe anemia show neutropenia.
  - Because DNA synthesis is impaired in all proliferating cells, granulocytic precursors also display nuclear-cytoplasmic asynchrony in the form of **giant metamyelocytes and band forms**.
- **Megakaryopoiesis:** Megakaryocytes are normal or increased in number. They may be abnormally large and may have bizarre, multilobate nuclei with open nuclear chromatin.
- **Bone marrow iron:** In pure vitamin B_{12}/folic acid deficiency bone marrow iron is moderately increased.

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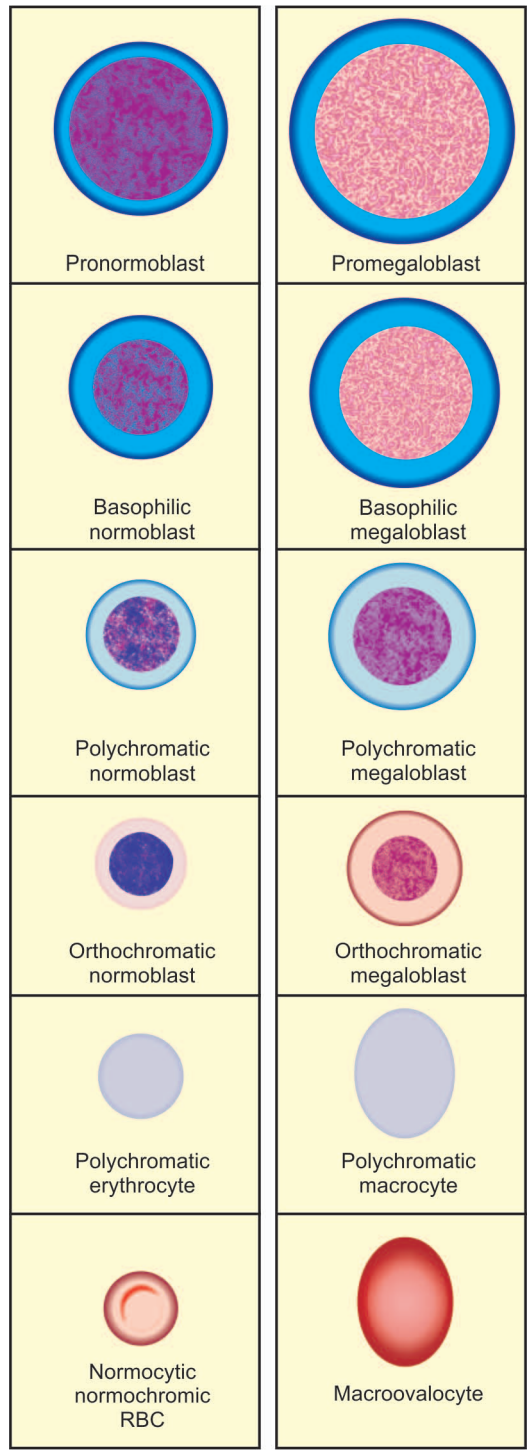


Fig. 4.8: Comparison of normoblastic erythropoiesis with megaloblastic type

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Common Biochemical Tests for Vitamin B_{12} and Folic Acid Deficiency

- The deoxyuridine suppression test: Deoxyuridine suppression test is a sensitive measure of deficiency of 5, 10-methylene THF, which occurs in both folic acid and vitamin B_{12} deficiency.
- Serum homocysteine: Their levels are increased.
- Serum bilirubin: Mild increase of serum bilirubin causes mild jaundice. This is due to hemolysis resulting from intramedullary death of megaloblasts in the bone marrow.
- Serum iron and ferritin: Both are increased in pure vitamin B_{12}/folic acid deficiency anemias.
- Plasma lactate dehydrogenase (LDH): Elevated often markedly.

ANEMIAS OF VITAMIN B_{12} DEFICIENCY

Causes of Vitamin B_{12} Deficiency (Table 4.2)

Decreased Intake

Vitamin B_{12} (cyanocobalamin) is mainly present in animal products. Vegetables and fruits contain very little vitamin B_{12}. Strict vegetarians who do not consume meat, eggs and milk are likely to develop vitamin B_{12} deficiency. Inadequate diet presents features of anemia after many years because of adequate vitamin reserves. Milk is a good source of vitamin B_{12}.

Impaired Absorption

The absorption of vitamin B_{12} can be impaired by disruption of any one of the steps involved in its absorption.

Gastric factors

- **Achlorhydria (deficiency of gastric acid) and loss of pepsin secretion:** The release of vitamin B_{12} from bound proteins in food requires pepsin and low pH produced by HCl.
- **Deficient IF:** Gastrectomy and in **pernicious anemia**, the intrinsic factor is inadequate or absent.

Intestinal factors

- **Loss of absorptive surface:** Conditions like ileal resection, diffuse ileal disease or Crohn disease result in defective absorption of intrinsic factor-vitamin B_{12} complex. Tropical sprue and nontropical sprue may lead to malabsorption of vitamins.
- **Bacterial or parasitic competition for vitamin B_{12}.**
  - **Bacterial competition:** Abnormal intestinal anatomy or surgical sequelae that results in stenotic areas known as blind loop or diverticulosis. In these conditions stasis of intestinal contents results in bacterial overgrowth which compete for vitamin B_{12}.
  - **Parasitic competition:** Fish tapeworm *Diphyllobothrium latum* infestation can induce a deficiency state by competing for vitamin B_{12}.

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Damage to the exocrine pancreas
With loss of exocrine pancreatic function, vitamin B_{12} cannot be released from R-binder-vitamin B_{12} complexes.

Increased Demand

The requirement for vitamin B_{12} can be so great as to produce a relative deficiency even with normal absorption. This may be observed in circumstances like pregnancy, hyperthyroidism, disseminated cancer and chronic infections.

Pernicious Anemia

Definition

Pernicious anemia (PA) is a chronic disease resulting from deficiency of intrinsic factor causing impaired absorption of vitamin B_{12} and eventually megaloblastic anemia.

Incidence

Pernicious anemia may occur in all racial groups but is very rare in India. A genetic predisposition is suspected, because of tendency to form antibodies against multiple self-antigens.

Age
PA is a disease of older age and generally presents in the fifth to eighth decades of life.

Sex
Females are more involved than males (F:M is 1.5: 1).

Etiopathogenesis

Pernicious anemia is an **autoimmune** disease which develops due to destruction of gastric mucosa. The evidences for autoimmune etiology are:

- Its association with other autoimmune diseases like Graves disease, Hashimoto thyroiditis and adrenalitis is well established.
- Microscopic examination of stomach shows damage to gastric parietal cells accompanied by dense infiltration by lymphocytes and plasma cells. These changes are mediated both by cellular and humoral immune reactions and cause ***chronic atrophic gastritis***.
- Response to steroids.
- Presence of autoantibodies in most of the patients. **Two major types** of autoantibodies are found.
  - **Anti-intrinsic factor (IF) antibody**
    - Type I (blocking) antibody: This antibody blocks the binding of vitamin B_{12} to IF and are present in 50-75% of the cases. It can be detected in both plasma and gastric juice.
    - Type II (binding) antibody: It attaches to the IF–vitamin B_{12} complex and prevent its binding to receptors in the ileum. It is present in about 40% of patients.

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- **Parietal cell (Type III) antibody:** It is directed against ATPase pump in parietal cells but is neither specific for PA nor other autoimmune disorders. It is found in 90% of patients with PA as well as in older patients with chronic nonspecific gastritis.

The autoimmune process starts with activation of CD4+ T cells which initiates injury to the gastric mucosa. The mucosal damage secondarily triggers the formation of autoantibodies, which exacerbate the damage to IF secreting parietal cells. Anemia develops when sufficient number of parietal cells are damaged (resulting in IF deficiency) along with depletion of vitamin B_{12} reserves. Thus, autoantibodies are not the primary cause of chronic atrophic gastritis but they are of diagnostic value.

Clinical Features (Fig. 4.9)

- **Onset:** Insidious and progresses slowly unless halted by therapy.
- **Classic triad of presentation:** Weakness, sore throat and paraesthesias.
- **Tongue:** Painful red **"beefy"** tongue due to glossitis and atrophy of papillae. The patient complains of loss of taste and appetite.
- **Peripheral neuropathy:** Glove and sock distribution of numbness or paraesthesia. This tingling begins in tips of toes and progresses proximally and is bilateral and symmetric.
- **Ataxia:** Ataxic, uncoordinated gait, impairment of vibration and position sense.
- **Atherosclerosis:** Serum homocysteine level is raised and is a risk factor for atherosclerosis and thrombosis.

Laboratory Findings (Fig. 4.9)

Blood and bone marrow

The changes in the bone marrow and blood are similar to those described earlier for all megaloblastic anemias (Refer page 31-36).

Morphology

Alimentary system: Abnormalities are regularly found in the tongue and stomach.

- **Atrophic glossitis:** The tongue appears shiny, glazed and beefy.
- Stomach:
  - **Diffuse chronic atrophic gastritis** is associated with impaired secretion of hydrochloric acid, pepsin and intrinsic factor.
    - The characteristic histological feature is the atrophy of the glands (mainly in the fundus), with loss of both chief cells and parietal cells. As the disease advances parietal cells disappear completely.
    - The nuclei of mucosal cells are increased in size and look similar to that of megaloblasts.
    - There is dense infiltration by lymphocytes and plasma cells. Severity of gastritis worsens with advancing age.
  - **Intestinal metaplasia**: The epithelium lining the glands is replaced by mucus-secreting goblet cells which resemble those lining the large intestine. This is a type of metaplasia and is known as intestinal metaplasia/**intestinalization**.

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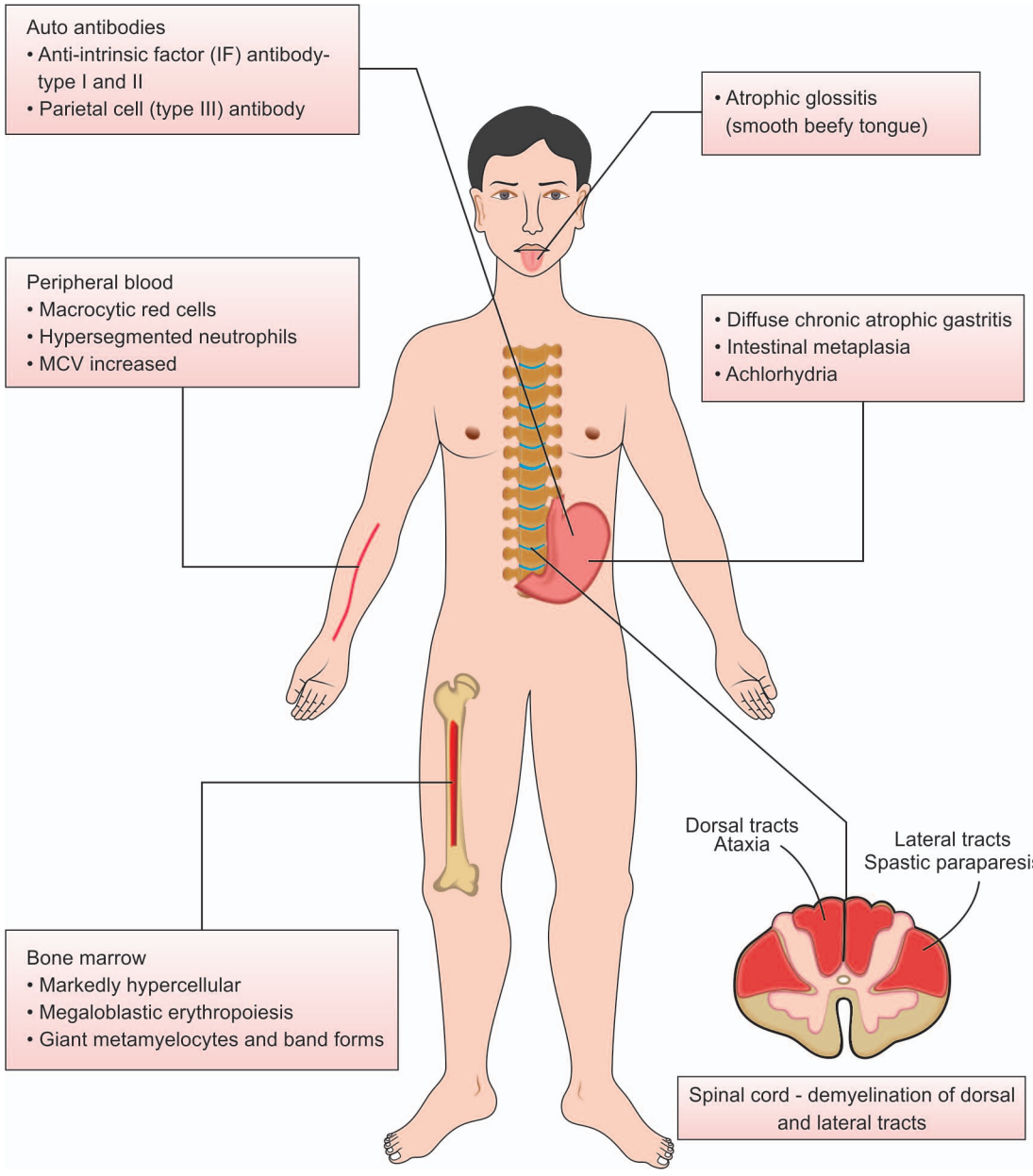


Fig. 4.9: Clinical features and laboratory findings in pernicious anemia

The gastric atrophy and metaplastic changes are due to autoimmune reaction and not due to deficiency of vitamin B_{12}. So, parenteral administration of vitamin B_{12} corrects the changes in the bone marrow, but not the gastric changes. Incidence of gastric cancer is higher in patients with pernicious anemia.

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Central nervous system: The lesions are found in 75% of all cases of severe pernicious anemia.

- **Demyelination in the dorsal and lateral tracts:** The spinal cord shows demyelination in the dorsal and lateral tracts. Demyelination of **dorsal** tracts causes sensory ataxia and that of **lateral** tracts gives rise to spastic paraparesis, and severe paraesthesia in the lower limbs.
- Because both sensory and motor pathways are involved, the term "subacute combined degeneration" is used to describe these neurologic changes found in vitamin B_{12} deficiency.

Biochemical Parameters

- **Diagnostic tests for vitamin B_{12} deficiency**
  - Serum vitamin B_{12} levels: **Decreased**
  - Serum methyl malonic acid: Increased
  - Urinary excretion of methylmalonic acid: Increased
  - Schilling test for vitamin B_{12} absorption: Radioactive vitamin B_{12} is used to assess the status of intrinsic factor (IF) and vitamin B_{12} and helps in distinguishing megaloblastic anemia due to IF deficiency (pernicious anemia) from other causes of vitamin B_{12} deficiency. It is diagnostic of PA but now very infrequently performed.
- **Specific diagnostic tests for pernicious anemia:**
  - Serum antibodies to intrinsic factor are highly specific for pernicious anemia. Their presence confirms the cause of vitamin B_{12} deficiency, rather than the presence or absence of cobalamin deficiency.
  - Achlorhydria with histamine/pentagastrin stimulation (Chapter 62).
  - Severe deficiency of intrinsic factor.

ANEMIA OF FOLATE DEFICIENCY

A deficiency of folic acid causes megaloblastic anemia similar to that of vitamin B_{12} deficiency. However, the neurologic changes seen in vitamin B_{12} deficiency do not occur.

Causes of Folic Acid Deficiency

The body's reserves of folate are relatively modest, and a deficiency can arise with months of negative balance. The major causes of folic acid deficiency are shown in Table 4.2.

Clinical Features

Similar to vitamin B_{12} deficiency anemia except the neurological symptoms.

Note: The hematologic symptoms of a vitamin B_{12} deficiency anemia also respond to folate therapy. However, folate does not prevent (and may even exacerbate) the progression of the neurologic deficits of the vitamin B_{12} deficiency. Always in megaloblastic anemias, before initiating therapy with folate, first exclude vitamin B_{12} deficiency.

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Laboratory Findings

Peripheral Blood

Similar to those mentioned under laboratory findings of megaloblastic anemia (Refer page 31-33).

Bone Marrow

Similar to those mentioned under laboratory findings of megaloblastic anemia (Refer page 33-35).

Biochemical Parameters

Similar to those mentioned under laboratory findings of megaloblastic anemia (Refer page 36).

Specific Tests for Folic Acid Deficiency

- **Serum folic acid levels: Decreased**
- **FIGLU in urine:** Formiminoglutamate is an intermediate product in the conversion of histidine to glutamate and is excessively excreted.

NONMEGALOBLASTIC CAUSES OF MACROCYTIC ANEMIAS

These are shown in Table 4.3

Table 4.3: Nonmegaloblastic causes of macrocytic anemias

Alcohol
Liver disease
Myxedema
Cytotoxic drugs
Myeloma

SUMMARY

- Megaloblastic anemias are characterized by impaired DNA synthesis and distinct RBC precursors megaloblasts in the bone marrow and macrocytic red cells in the peripheral blood.
- The megaloblasts are large, abnormal precursors of RBCs, which show nuclear cytoplasmic asynchrony.
- Two major causes are deficiency of vitamin B_{12} and folic acid.
- Vitamin B_{12} deficiency results from inadequate intake, increased demand or inadequate absorption. The absorption requires intrinsic factor secreted by parietal cells of the stomach.
- Pernicious anemia is an autoimmune disorder characterized by deficiency of intrinsic factor (IF).

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- Folic acid deficiency may result from dietary deficiency, impaired absorption, increased demand or increased loss.
- The characteristic findings include oval macrocytes and hypersegmented neutrophils in the peripheral blood. Bone marrow is hyperplastic and shows megaloblasts.

SELF-ASSESSMENT EXERCISES

I. Essays

1. Mention the causes of megaloblastic anemia. Discuss the laboratory diagnosis of megaloblastic anemia
2. Discuss the etiopathogenesis morphology and laboratory findings in pernicious anemia

II. Short Notes

1. Megaloblast
2. Megaloblastic bone marrow
3. Differences between normoblast and megaloblast
4. Enumerate the causes of macrocytic anemia
5. Hematological and biochemical changes in megaloblastic anemia
6. Peripheral smear and bone marrow findings in megaloblastic anemia

III. Multiple Choice Questions

1. **The condition associated with increased demand of vitamin B_{12} is**
   A. Aplastic anemia
   B. Hypothyroidism
   C. Pregnancy
   D. Splenectomy
2. **Consequence of vitamin B_{12} deficiency is impaired**
   A. Absorption of iron
   B. DNA synthesis
   C. Folic acid synthesis
   D. Production of intrinsic factor
3. **The following does NOT cause vitamin B_{12} deficiency**
   A. Pernicious anemia
   B. Fish tapeworm infestation
   C. Ileal resection
   D. Colectomy
4. **Vitamin B_{12}-IF complex is absorbed in**
   A. First part of duodenum
   B. Second part of duodenum
   C. Jejunum
   D. Ileum

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5. **The intrinsic factor is required for the absorption of**
   A. Iron
   B. Zinc
   C. Folic acid
   D. Vitamin B_{12}
6. **Deficiency of intrinsic factor may be caused by**
   A. Gastrectomy
   B. Crohn disease
   C. Malabsorption syndrome
   D. Large bowel resection
7. **The best and earliest hematological finding in megaloblastic anemia is**
   A. Decreased hemoglobin
   B. Decreased hematocrit
   C. Hypersegmented neutrophils
   D. Poikilocytosis
8. **The peripheral blood smear in megaloblastic anemia shows following features EXCEPT**
   A. Macroovalocytes
   B. Howell-Jolly bodies
   C. Increased reticulocytes
   D. Hypersegmented neutrophils
9. **RBCs in megaloblastic anemia show following changes in the peripheral smear EXCEPT**
   A. Basophilic stippling
   B. Cabot rings
   C. Howell-Jolly bodies
   D. Heinz bodies
10. **The neutrophils in peripheral blood smear of megaloblastic anemia show**
    A. Shift to the left
    B. Howell-Jolly bodies
    C. Toxic granules in the cytoplasm
    D. Hypersegmentation
11. **The bone marrow of megaloblastic anemia shows**
    A. Plasma cells and giant myelocytes
    B. Hypersegmented neutrophils and thrombocytosis
    C. Megaloblasts and micromegakaryocyte
    D. Giant metamyelocytes and ineffective erythropoiesis
12. **The following are the features of megaloblast EXCEPT**
    A. Larger than normoblast
    B. Nuclei have more open sieve-like chromatin
    C. Cytoplasmic maturation lags behind nuclear maturation
    D. Marrow shows increased proportion of more primitive erythroid cells
13. **Following are laboratory findings in megaloblastic anemia EXCEPT**
    A. Increased number of platelets
    B. Increased mean corpuscular volume
    C. Megaloblastic precursors in bone marrow
    D. Increased serum LDH

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14. Deficiency of the 'intrinsic factor' causes
A. Sickle cell anemia
B. Pernicious anemia
C. Thalassemia
D. Aplastic anemia

15. Schilling test is positive in
A. Pernicious anemia
B. Aplastic anemia
C. Sickle cell anemia
D. Hereditary spherocytosis

16. Folic acid normally circulates in plasma as
A. Monoglutamate
B. Polyglutamate
C. Methylmalonic acid
D. 5-methy tetrahydrofolate

17. Excessive excretion of formiminoglutamic acid (FIGLU) in urine is found in
A. Vitamin B_{12} deficiency
B. Niacin deficiency
C. Folic acid deficiency
D. Intravascular hemolysis

18. The following findings is NOT a major criteria for diagnosis of pernicious anemia
A. Megaloblastic anemia
B. Low serum vitamin B_{12}
C. Anti-intrinsic factor (IF) antibody
D. Gastric parietal cell antibody

19. Folic acid deficiency can be caused by
A. Alcoholism
B. Pure vegetarian diet
C. Chronic blood loss
D. Excessive intake of iron

20. The type of anemia associated with degeneration of myelin in dorsal and lateral tracts of spinal cord is
A. Immunohemolytic anemia
B. Pernicious anemia
C. Aplastic anemia
D. Sickle cell anemia

ANSWERS

1. C	**2.** B	**3.** D	**4.** D	**5.** D	**6.** A	**7.** C	**8.** C
9. D	**10.** D	**11.** D	**12.** C	**13.** A	**14.** B	**15.** A	**16.** D
17. C	**18.** D	**19.** A	**20.** B				

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CHAPTER 5 Introduction and Classification of Hemolytic Anemia

CHAPTER OUTLINE

- Introduction
- Hemolytic Anemia
- Classification of Hemolytic Anemias

INTRODUCTION

Function of RBC: The major function of red blood cells (RBCs) is to carry oxygen to the tissues and return carbon dioxide from tissues to the lungs. This important function is carried out by conjugated protein ***hemoglobin (Hb),*** which is the major component of RBC. Hemoglobin imparts red color to the RBCs.

Hemoglobin Structure

- A hemoglobin molecule is composed of iron containing pigment called **heme** and protein **globin**. About 65-70% of hemoglobin is synthesized in normoblasts and 30-35% is synthesized at the reticulocyte stage.
  - Each molecule of **heme** consists of protoporphyrin with an iron (Fe^{++}) atom. Heme synthesis occurs mainly in the mitochondria of normoblasts.
  - Each **globin** chain is made up of **two pairs of unlike polypeptide** chains bound to a heme molecule. Each polypeptide chain is designated by a Greek letter.
- Different types of globin chains are produced during the embryonic, fetal, and postnatal (adult) stages of development (Fig. 5.1).

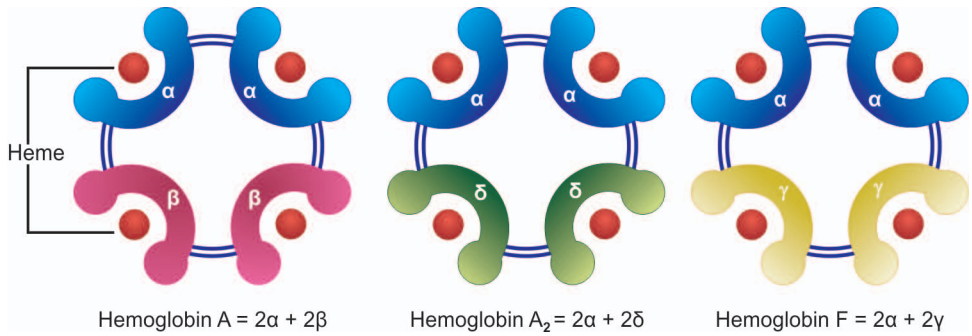


Fig. 5.1: Composition of different hemoglobins

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- In adults four types of globin chains are produced namely alpha, beta, gamma and delta (**α, β, γ, δ**). All normal hemoglobins contain α chain.
- **Hemoglobin A (HbA)** is composed of two α-globin chains and two α-globin chains **(α_2 β_2)**. It normally represents more than 95% of the hemoglobin in adult RBCs. Fetal hemoglobin is replaced by adult hemoglobin during the first year of life (hemoglobin switching).
- **Hemoglobin A_2 (HbA_2)** is normally found at low levels in adults and contains 2 α and 2 δ chains (α_2 δ_2).
- **Fetal hemoglobin (Hb F)** contains two α-globin chains and two γ-globin chains (**α_2 γ_2**). It is the major hemoglobin during fetal development and is normally found in low levels in adults.

Hemoglobin molecules containing different globin chains can be distinguished from one another by electrophoresis or liquid chromatography.

Note: Glycosylated hemoglobin (HbA_{1c}): HbA_1 is a minor component of adult hemoglobin (HbA) and its important subgroup is HbA_{1c}. HbA_{1c} has an irreversibly attached glucose and is known as glycosylated hemoglobin. HbA_{1c} synthesis is dependent on glucose level in the blood. If young RBCs are exposed to extremely high blood glucose (more than 400 mg/dL) for several hours, HbA_{1c} level increases. Thus, in patients with diabetes mellitus, it is useful in assessing the blood glucose control, higher the count poorer is the control.

Acquired Chemically Modified Hemoglobin Derivatives

- **Methemoglobin (Hi):** It is a hemoglobin in which the iron is in ferric form (in normal Hb it is in ferrous form) and unable to combine with oxygen. Increased amount of methemoglobin in the RBCs is called methemoglobinemia. It may be hereditary or acquired. The drugs and chemicals are the main acquired causes.
- **Sulfhemoglobin (SHb):** It is a formed by the irreversible oxidation of hemoglobin by certain drugs and chemicals (sulfonamides, phenacetin). It is ineffective for oxygen transport.
- **Carboxyhemoglobin (HbCO):** It is formed when hemoglobin is exposed to carbon monoxide gas. Carbon monoxide is known as silent killer because the victims exposed to this colorless and odorless gas quickly develop hypoxic death. Normal blood contains about 0.5% carboxyhemoglobin and it is increased in hemolytic anemia.

Defects in Hemoglobin

Hemoglobin defects may be in the form of production of abnormal (qualitative) or reduced production (quantitative) of normal hemoglobin. The term hemoglobinopathy unless specified usually indicates a qualitative hereditary disorder.

- ***Qualitative defect in (abnormal) hemoglobins:*** Genetic mutations in the α-globin or β-globin locus may result in the production of abnormal hemoglobin with a different amino

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acid composition (e.g. sickle cell anemia). The heme portion of the molecule is normal. Though the abnormal variant hemoglobin may be functionally normal, it may have physical or physiologic properties that differ from those of a normal hemoglobin molecule.

- ***Quantitative defect in hemoglobins:*** A second type of genetic mutation in the globin loci (e.g. thalassemia) is characterized by a quantitative reduction in the synthesis of α-globin or β-globin chains and a net reduction in the formation of hemoglobin.

HEMOLYTIC ANEMIA

Definition

Hemolytic anemias are defined as anemias that result due to increase in the rate of red cell destruction.

- Normal life span of red cells is approximately 120 days and premature or increased rate of destruction of circulating RBCs is known as hemolysis. The resulting anemias are hemolytic anemias in which a major feature is reduction in red cell life span which may be as low as 10-15 days.
- If the compensatory marrow regeneration (by increased erythropoiesis) is adequate, patient does not manifest anemia and such a case is referred to as compensated hemolytic disease.
- However, in moderate to marked hemolysis compensatory mechanisms are not adequate and patients develop clinical features of hemolytic anemias.

Mechanism and Consequences of Hemolysis

These may be divided into three major categories.

A. Features of Increased Red Cell Destruction

Older RBCs at the end of normal life span of 120 days are termed senescent. The physiological destruction of senescent RBCs takes place mainly (80-90%) extravascularly within the mononuclear phagocytic (RE) cells of the spleen, liver and bone marrow. In hemolytic anemia, there is excessive destruction of RBCs (hemolysis). Increased hemolysis results in accumulation of hemoglobin degradation products released from RBC lysis. They result in:

- Increased unconjugated bilirubin in blood: **Jaundice.**
- **Increased stercobilinogen** in stool causing dark colored stool. Excessive bilirubin excreted by the liver into the gastrointestinal tract leads to increased formation and fecal excretion of stercobilinogen and stercobilin.
- Increased urobilinogen in urine leading to **high colored urine**.
- **Increased iron stores**—iron released from heme is stored in bone marrow.

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Site of RBCs destruction

The site of destruction of red cells may be extravascular or intravascular.

Extravascular red cell destruction (Fig. 5.2)

- It is the major mechanism of destruction of normal red blood cells. It is also the major pathogenetic mechanism of hemolysis in a variety of hemolytic anemias.
- Premature destruction of red cells occurs within the mononuclear phagocytes of the reticuloendothelial (RE) system. The contents of red cells are not detected in the plasma because it is degraded inside the phagocytic macrophages-hence referred to as **extravascular hemolysis** (*macrophage-mediated hemolysis*). Extravascular hemolysis in the RE system leads to "work" hyperplasia of organs like spleen, liver and bone marrow.
- Red cells should undergo extreme alterations in shape for its successful passage in the splenic sinusoids. This is facilitated in normal RBC because of its characteristic cell membrane.
- In extravascular hemolysis red cells are phagocytosed either because RBCs are rendered "foreign" or become less deformable, which makes their passage difficult in splenic sinusoids. Most of the pathologic destruction of RBCs occurs mainly in the spleen and results in splenomegaly.
- In the RE system, the destroyed or lysed senescent red cells release hemoglobin. Hemoglobin is broken down into heme and globin. Globin is further degraded into amino acids and returned to the amino acid pool. Heme part is split into iron (which is reutilized) and protoporphyrin. Protoporphyrin is converted into bilirubin which binds to serum albumin (unconjugated) and circulates in the blood.
- Conjugation of bilirubin takes place in liver and conjugated bilirubin is excreted via bile into the intestine.
- Conjugated bilirubin is degraded by gut bacteria into various water-soluble colorless compounds, collectively known as urobilinogen. Most of the urobilinogen is oxidized further to stercobilinogen and excreted in feces as stercobilin.
- About 20% of the urobilinogens are reabsorbed from the intestine (ileum and colon), returned to the liver and re-cycled directly into the bile again. A small amount of this reabsorbed urobilinogen is found in the blood circulation and is filtered by the renal glomerulus and excreted in the urine as urobilinogen and urobilin.

  The **characteristic findings of extravascular hemolysis** are:
  - **Anemia:** Excessive destruction of blood cells causes anemia.
  - **Jaundice:** Excessive production of bilirubin causes jaundice.
  - **Splenomegaly**: Hyperplasia of the mononuclear phagocyte system often leads to splenomegaly. As most of extravascular hemolysis occurs in the spleen, splenectomy is beneficial in most patients.
  - **With extravascular hemolysis, hemoglobinemia and hemoglobinuria are not observed**.

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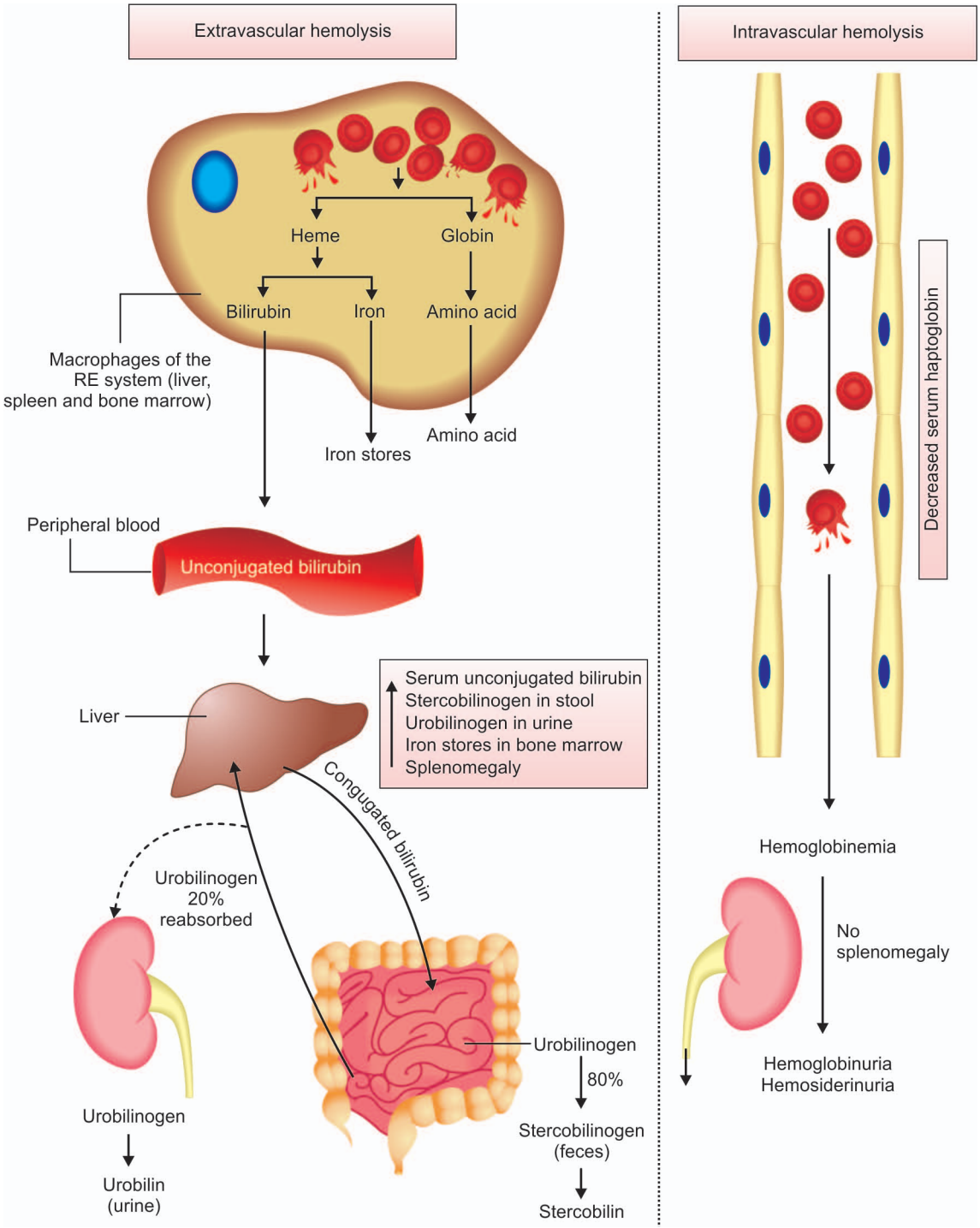


Fig. 5.2: Mechanisms and consequences of extravascular and intravascular hemolysis

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Intravascular red cell destruction (Fig. 5.2)

Intravascular hemolysis is the minor pathway of normal red cell destruction. It can be a major pathological process in few hemolytic anemias. The hemolysis occurs within the circulation with release of red blood cell contents, including hemoglobin directly into the plasma. Intravascular hemolysis may be caused by mechanical injury (e.g. trauma by heart valves), intracellular parasites (e.g. malarial parasites), complement fixation (e.g. binding of antibodies against red cell antigens) or exogenous toxic injury (e.g. clostridial sepsis).

The **characteristic findings of intravascular hemolysis** are:

1. **Hemoglobinemia:** Intravascular hemolysis releases free hemoglobin which binds to haptoglobin in plasma. The hemoglobin and haptoglobin complex formed is rapidly cleared by the mononuclear phagocyte system, thus preventing hemoglobin excretion into the urine.
2. **Decreased serum haptoglobin: It is characteristic of intravascular hemolysis.**
3. **Hemoglobinuria:** When the plasma hemoglobin exceeds the haptoglobin binding capacity, free elevated hemoglobin is detected in the urine (hemoglobinuria). Hemoglobinuria may impart dark red or brown (cola/root beer) color to the urine because of its oxidation to brown colored methemoglobin.
4. **Hemosiderinuria:** In hemoglobinuria of chronic intravascular hemolysis, iron released from hemoglobin may be reabsorbed by the renal proximal tubular cells. Iron accumulates within tubular cells as hemosiderin and can be detected in the urine sediment by Prussian blue reaction.
5. **Anemia:** Excessive destruction of blood cells causes anemia.
6. **Jaundice:** Excessive production of bilirubin causes jaundice and since it is due to breakdown of red cells, it is known as hemolytic or prehepatic jaundice.
7. Unlike in extravascular hemolysis, **splenomegaly is not found**.
8. Plasma lactate dehydrogenase **(LDH):** Increased

Differences between extravascular and intravascular hemolysis are shown in Table 5.1.

Table 5.1: Differences between extravascular and intravascular hemolysis

	Extravascular hemolysis	*Intravascular hemolysis*
Site of hemolysis	Mononuclear phagocytes of RE system (spleen, bone marrow)	Within the circulation
Diseases causing hemolysis	Thalassemia, sickle cell anemia	PNH, G6PD deficiency
Serum haptoglobin	Normal	Decreased
Hemoglobinemia	Not seen	Positive
Hemoglobinuria	Not seen	Positive
Hemosiderinuria	Not seen	Positive
Serum bilirubin (unconjugated)	Moderately elevated	Mildly elevated
Splenomegaly	Usual	Uncommon

PNH = Paroxysmal nocturnal hemoglobinuria; G6PD = Glucose-6-phosphate dehydrogenase

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B. Features of Increased Red Cell Production

As a compensatory mechanism to hemolysis, there is increased production of red cells.

- Anemia and resultant tissue hypoxia causes increased erythropoietin production by kidney.
- Increased erythropoietin stimulates bone marrow and produces **compensatory erythroid hyperplasia.**
- Bone marrow hyperplasia leads to **increased reticulocytes** in the peripheral blood.

C. Damaged Red Cells

- Morphological features: These include presence of microspherocytes, elliptocytes, red cell fragments, etc. in the peripheral blood.
- Red cell survival is shortened.

Clinical Features

Clinical signs and symptoms of hemolytic anemia depend upon the severity, duration and type of hemolytic anemia. Common clinical manifestations in chronic hemolytic anemias are:

- **Anemia:** Anemia results in pallor of varying degree and is better appreciated in mucous membranes like conjunctiva. The general symptoms of anemia are fatigue, dyspnea on exertion and tachycardia.
- **Jaundice:** Increased bilirubin gives rise to yellowish discoloration of sclera and skin known as jaundice and of plasma referred to as icterus. Jaundice can develop due to conditions other than hemolysis. Jaundice due to hemolysis is known as hemolytic jaundice and since it is predominantly due to unconjugated bilirubinemia, it is also referred to as prehepatic jaundice. In most cases jaundice is mild. In G6PD deficiency, hemolysis being periodical, jaundice appears during crisis.
- **Splenomegaly:** Splenomegaly is due to increased red cell destruction by phagocytic macrophages (work hyperplasia) of the spleen and also due to foci of extramedullary hematopoiesis. In sickle cell anemia, repeated episodes of splenic infarcts result in autosplenectomy.
- **Gall stones (cholelithiasis):** Chronic hemolytic anemia resulting in excessive excretion of bilirubin in the bile promotes the formation of pigment gallstones. All young patients with gallstones should be investigated to rule out hemolytic anemias.
- **Skeletal deformities:** Chronic hemolytic anemia causes persistent marked bone marrow hyperplasia. In children since bones are still developing, it may cause bone deformities. Typical thalassemic facies is characterized by widening of diploe of skull (frontal and parietal bones) and maxilla causing frontal bossing, prominent cheek bones and wide spacing of teeth.
- **Leg ulcers:** Leg ulcers manifest in adult males especially in patients with hereditary spherocytosis and sickle cell anemia secondary to stasis.

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Laboratory Findings in Hemolytic Anemias

Peripheral Blood

- **Hemoglobin:** It is decreased and varies with the type and duration of hemolytic anemia.
- **Reticulocyte count:** Increased.
- **Peripheral smear:** It is the most important investigation in hemolytic anemia. The following findings alone or in combination suggest hemolysis:
  - **RBCs**
    - They show markedly increased **reticulocyte count**, which appear as large **polychromatophilic** red blood cells in the peripheral blood.
    - **Nucleated RBCs:** Moderate to marked hemolysis results in appearance of nucleated red cells, mostly late normoblasts.
    - Red cell morphology provides a clue to the underlying hemolytic disorder like:
      - Spherocyte (see Fig. 6.3A and B), sickle cell (see Fig. 8.4A and B), target cell (see Fig. 7.4A and B), acanthocyte (see Fig. 42.3B), schistocyte (see Fig. 42.3A), malarial parasite (see Fig. 42.7A)
  - **WBCs: Neutrophilia** with shift to left and presence of metamyelocytes and myelocytes is seen in active hemolysis.
  - **Platelets:** In acute hemolysis, there is **thrombocytosis** with numerous large platelets.

Bone Marrow

Bone marrow examination is usually not necessary for the diagnosis of hemolytic anemia.

- **Cellularity:** Due to erythroid hyperplasia, the overall cellularity of the bone marrow is **increased.**
- **Erythropoiesis:** Anemia with tissue hypoxia stimulates increased production of erythropoietin, which causes erythroid hyperplasia in bone marrow. Erythroid hyperplasia is the morphological hallmark of various hemolytic anemias and is characterized by **increased number of erythroid precursors (normoblasts) in the marrow.**
- **M:E ratio:** The myeloid-erythroid ratio is decreased with a reversal ranging from 1:1 to 1:6.

Extramedullary Hematopoiesis

- Extramedullary hematopoiesis develops when marrow erythroid hyperplasia is not able to ameliorate moderate to severe anemia because of **"ineffective erythropoiesis".** The term ineffective erythropoiesis is used when developing erythroid precursors die within the marrow without producing mature cells**.**
- **Extramedullary hematopoiesis** can appear in the liver, spleen and lymph nodes.
- Mostly found in hereditary hemolytic anemias like thalassemia and sickle cell anemia.

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Other Laboratory Findings

- Increased serum unconjugated (indirect) bilirubin.
- Increased urine urobilinogen.
- Raised fecal stercobilinogen.
- Decreased life span of red cells.
- Absent/diminished serum haptoglobin, which binds hemoglobin.
- Increased serum LDH levels in intravascular hemolysis.

CLASSIFICATION OF HEMOLYTIC ANEMIAS

The hemolytic anemias are classified (Table 5.2) in a variety of ways.

- **Location of hemolysis:** Depending upon the site of red cell destruction it can be classified as: **intravascular and extravascular** hemolytic disorders. However, since disorders with predominantly intravascular hemolysis are quite uncommon, this classification is not entirely satisfactory.
- **Source of defect causing hemolysis**: Pathogenetic classification is based on the underlying cause of red cell destruction into those due to a defect **intrinsic** to the red cell itself (**intracorpuscular defect)** and those due to an abnormality outside the red cell **(extracorpuscular mechanism).**
- **Mode of onset:** According to mode of onset, it may be classified as **hereditary and acquired disorders.** In general, hereditary disorders are due to intrinsic defects and the acquired disorders due to extrinsic factors such as autoantibodies.

Table 5.2: Classification and causes of hemolytic anemia

INTRACORPUSCULAR DEFECT

A. Inherited genetic defects

- **Red cell membrane defects**
  - Hereditary spherocytosis
  - Hereditary elliptocytosis
  - Abetalipoproteinemia
- **Red cell enzyme deficiencies**
  - Hexose monophosphate shunt enzyme deficiencies
    - Glucose-6-phosphate dehydrogenase deficiency (G6PD)
  - Glycolytic enzyme deficiencies
    - Pyruvate kinase deficiency
    - Hexokinase deficiency
- **Hemoglobin abnormalities**
  - Deficient globin synthesis
    - Thalassemia syndromes: α thalassemia, β thalassemia
  - Structurally abnormal globins (hemoglobinopathies)
    - Sickle cell disease, unstable hemoglobins

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B. Acquired genetic defects
- Deficiency of phosphatidylinositol-linked glycoproteins
  - Paroxysmal nocturnal hemoglobinuria

EXTRACORPUSCULAR DEFECT

A. Immunohemolytic anemias (antibody-mediated destruction)
- **Autoimmune hemolytic anemias**
  - Warm antibody type (IgG antibodies active at 37°C)
    - Primary (idiopathic)
    - Secondary: Autoimmune disorders (SLE), drugs, lymphomas
  - Cold agglutinin type (IgM antibodies active at 4°C to 18°C)
    - Acute: Mycoplasmal infection, infectious mononucleosis
    - Chronic: Idiopathic, lymphomas
  - Cold hemolysin type (Donath-Landsteiner antibodies)
- Alloimmune hemolytic anemias
  - Hemolytic disease of the newborn (HDN)
  - Hemolytic transfusion reactions: Mismatched blood transfusion

B. Fragmentation syndromes/mechanical trauma
- Microangiopathic hemolytic anemias
  - Hemolytic uremic syndrome
  - Thrombotic thrombocytopenic purpura
  - Disseminated intravascular coagulation
- Macroangiopathic hemolytic anemias
  - Prosthetic cardiac valves, synthetic vascular graft

C. Drugs, chemicals and toxins
- Drugs: Oxidant drugs, primaquine, dapsone, etc.
- Chemicals: Naphthalene, nitrites, nitrates, oxidizing chemicals, etc
- Toxins: Snake venom, lead poisoning, clostridial sepsis

D. Sequestration: Hypersplenism

E. Infections of RBCs: Malaria, babesiosis

SLE = Systemic lupus erythematosus

SUMMARY

- Hemoglobin is composed of four heme molecules and two pairs of globin chains.
- Hemolytic anemias are characterized by increased destruction of red blood cells associated with compensatory erythroid hyperplasia of the bone marrow.
- Anemia develops when the hyperplasia of the marrow is not able to compensate for the increased destruction of red blood cells.
- Hemolytic anemias can be classified as intravascular and extravascular, or intracorpuscular and extracorpuscular or hereditary and acquired.
- Laboratory findings show increased reticulocyte count, hyperbilirubinemia and compensatory erythroid hyperplasia in the marrow. Peripheral smear examination can point out the intravascular or extravascular etiological factor.

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SELF-ASSESSMENT EXERCISES

I. Essay

Classify hemolytic anemias. Discuss the laboratory investigations in a case of hemolytic anemias.

II. Short Notes

1. Enumerate the causes of hemolytic anemia.
2. Differences between intravascular and extravascular hemolysis.

III. Multiple choice questions

1. The feature NOT seen in hemolytic anemia is
A. Increased urobilinogen in urine
B. Increased serum bilirubin
C. Decreased iron stores
D. Increased stercobilinogen in stool

2. Following features are characteristic of intravascular red cell destruction EXCEPT
A. Hemoglobinemia
B. Hemoglobinuria
C. Hemosiderinuria
D. Increased haptoglobin

3. The feature indicative of extravascular red cell destruction is
A. Hemoglobinemia
B. Hemoglobinuria
C. Splenomegaly
D. Decreased haptoglobin

4. Hemolytic anemia is characterized by all EXCEPT
A. Increased reticulocytes
B. Erythroid hyperplasia
C. Thrombocytopenia
D. Increased erythropoietin levels

5. The following is NOT a feature of hemolytic anemias
A. Accumulation of products of hemoglobin
B. A shortened red cell life span
C. Elevated erythropoietin levels
D. Defective DNA synthesis

6. Condition characterized by increased stercobilinogen in stool is
A. Aplastic anemia
B. Hemolytic anemia
C. Alcoholic liver disease
D. Complete biliary tract obstruction

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7. **The type of jaundice associated with increased urobilinogen without bilirubin in urine is**
   A. Acholuric
   B. Obstructive
   C. Hepatitis associated
   D. HIV associated
8. **The common clinical feature of hemolytic anemia is**
   A. Pica
   B. Jaundice
   C. Renal stones
   D. Neurological symptoms

ANSWERS

1. C **2.** D **3.** C **4.** C **5.** D **6.** B **7.** A **8.** B

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CHAPTER 6
Hemolytic Anemias due to Red Cell Membrane Disorders

CHAPTER OUTLINE

- Introduction
- Hereditary Spherocytosis
- Hereditary Elliptocytosis

INTRODUCTION

Structure of the Red Cell Membrane and Cytoskeleton (Fig. 6.1)

Red blood cell's normal biconcave shape and its remarkable elasticity or flexibility are due to its specialized membrane cytoskeleton made up of various proteins. This specialized membrane skeleton (cytoskeletal network) lies closely opposed to the internal surface of the plasma membrane. Changes in this membrane-cytoskeletal unit lead to increased cell rigidity and premature destruction of circulating red blood cells. Cytoskeletal proteins depending on their interaction with surface membrane proteins can be divided into two categories namely, **vertical** and **horizontal** interactions.

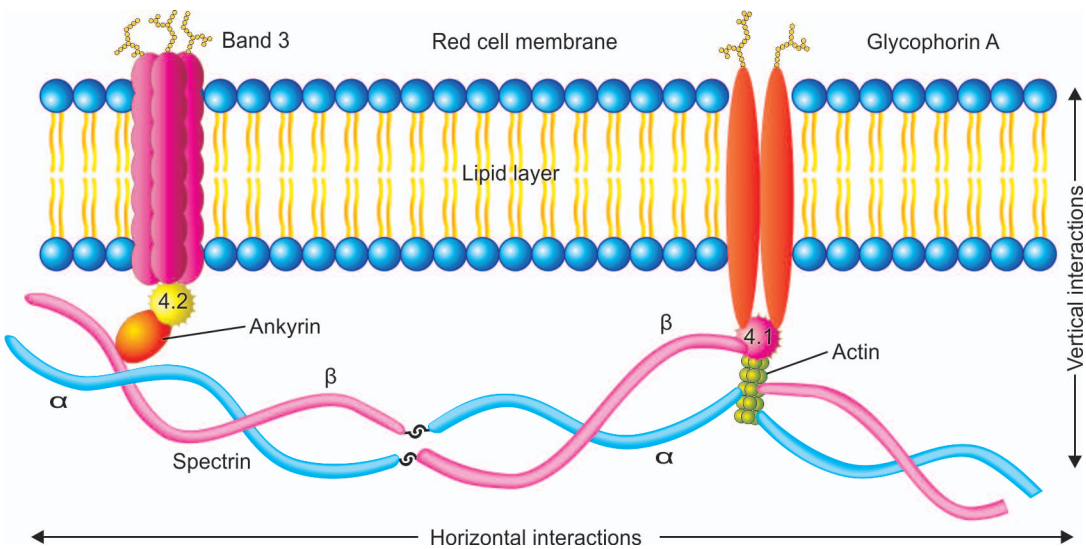


Fig. 6.1: Structure of the red cell membrane

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- **Horizontal interactions** are parallel to the plane of cell membrane and form stress – supporting network. Their chief protein components are:
  - **Spectrin,** which consists of two polypeptide chains, α and β, and with their head-to-head association forming tetramers, while the tails bind to actin oligomers.
  - Each **actin** oligomer can bind several spectrin tetramers. This **spectrin-actin skeleton** is connected to the cell membrane by two distinct vertical interactions.
- **Vertical interactions** are perpendicular to the red blood cell membrane and are connected to the inner surface of it. They stabilize the cell membrane.
  - There are two types of vertical interactions which connect transmembrane proteins to horizontal interaction (spectrin-actin skeleton) through linking proteins. The transmembrane proteins are band 3 and glycophorin A, which act as receptors/ channels.
  - In the first interaction, spectrin binds the transmembrane ion transporter **band 3**, through the linking proteins **ankyrin** and **protein 4.2**.
  - The second interaction consists of binding tail of spectrin-actin skeleton to another transmembrane protein, **glycophorin A**, involving linking **protein 4.1**.

Of all the membrane defects, hereditary spherocytosis (HS) is the commonest inherited hemolytic anemia.

HEREDITARY SPHEROCYTOSIS

Definition

Hereditary spherocytosis (HS) is a rare inherited hemolytic anemia resulting from the defect in the red cell membrane.

Etiopathogenesis

- Hereditary spherocytosis is characterized by defect in the RBC membrane due to deficiency of cytoskeleton protein.
- The defect in red cell membrane is caused by various mutations. The most common mutations involve ankyrin, band 3, spectrin, or band protein 4.2, the proteins involved in the first of the two tethering vertical interactions.

Mechanism of Hemolysis in HS (Fig. 6.2)

- Normally the red cells must undergo extreme deformation while passing through the sinusoids of the spleen.
- Initially in hereditary spherocytosis, young HS red cells are normal in shape. But as they age, there is loss of membrane fragments in the circulation.
- With the loss of membrane, the cells become small and assume a spherical shape and are known as **spherocytes.**
- These spherocytes are rigid, inflexible and less deformable and hence, are trapped in the spleen.

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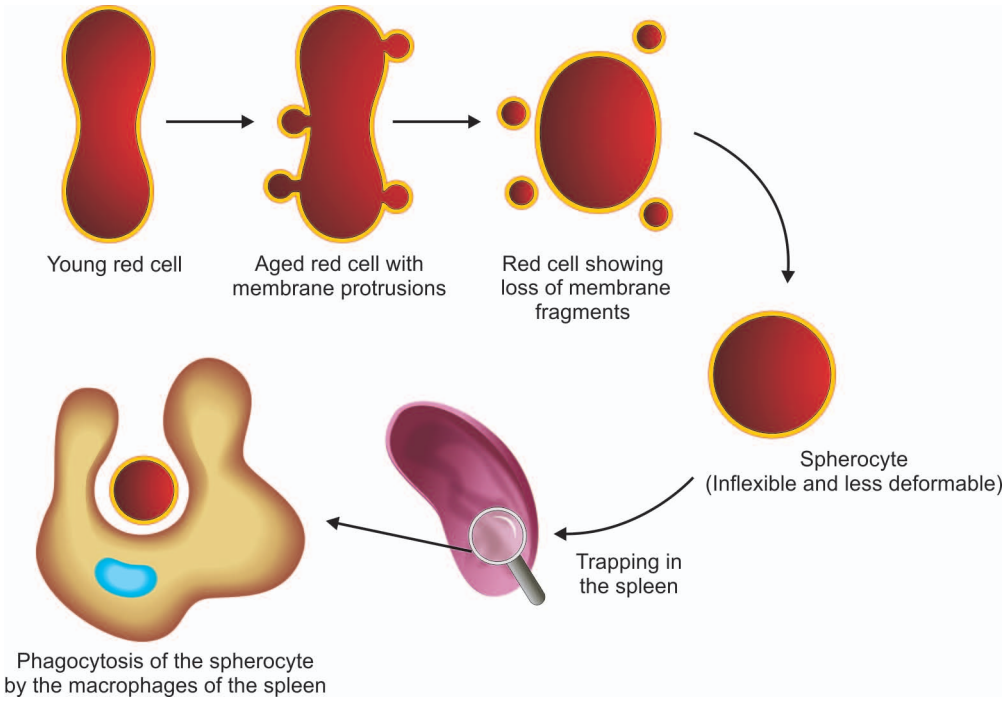


Fig. 6.2: Mechanism of hemolysis in hereditary spherocytosis

- In the spleen spherocytes undergo phagocytosis and destruction by RE cells resulting in extravascular hemolysis.
- Splenectomy reduces the premature destruction of spherocytes and corrects anemia (raises Hb) but the spherocytes persist.
- The affected red cells of hereditary spherocytosis have decreased lifespan of as low as 10 to 20 days as compared to normal 120 days.

Clinical Features

The clinical features are variable and are those of chronic extravascular hemolysis.

- **Age:** Disease may present during anytime from the neonatal period to adulthood. In some, the disease is asymptomatic because the destruction of red cells are compensated by erythroid hyperplasia.
- **Family history:** Most (75%) cases of HS are inherited as autosomal dominant trait.
- **Anemia:** Anemia is usually mild to moderate.
- **Jaundice:** Intermittent attacks of jaundice are the usual clinical presentation, which may be precipitated by pregnancy, fatigue, or infection.
- **Splenomegaly:** Moderate splenic enlargement (500 to 1000 gm) is characteristic and constant feature. Splenectomy corrects the anemia and its complications, but increases the risk of sepsis.

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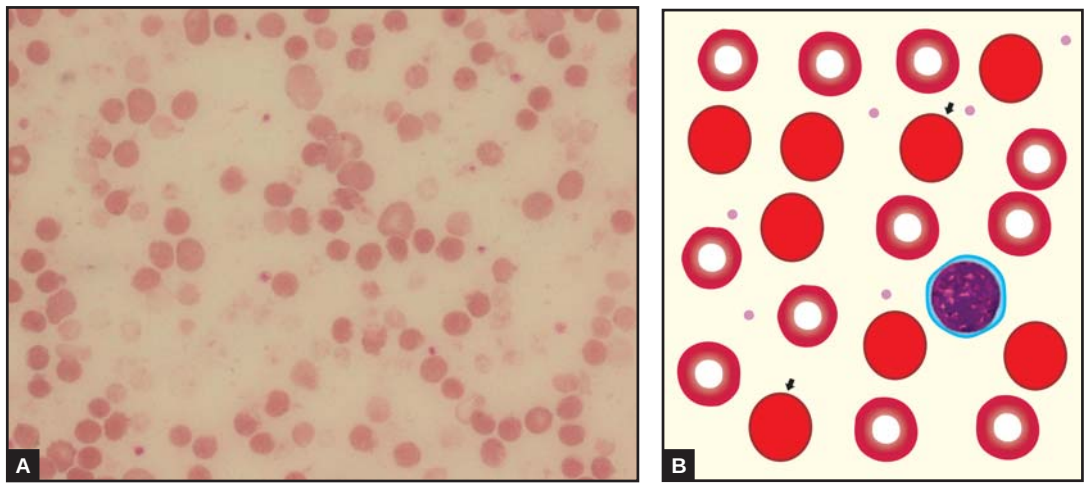
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- **Gallstones:** Pigment gallstones may be observed (because of high turnover of heme), in first and second decades of life.
- **Aplastic crises:** Sometimes HS presents with aplastic crisis, triggered by an acute parvovirus infection. Parvovirus infects and kills red cell progenitors, causing sudden cessation of red cell production. *Rarely, hemolytic crisis* is produced.

Laboratory Findings

Peripheral Blood

- **Hemoglobin**: Decreased and the level depends upon the degree of hemolysis.
- **Red cell indices**
  - **MCV is decreased**
  - **MCHC increased** due to dehydration caused by the loss of K^+ and H_2O.
- **Peripheral smear:** Peripheral smear examination is very important in arriving at a diagnosis (Figs 6.3A and B).



Figs 6.3A and B: Peripheral blood smear with numerous spherocytes (arrows)

  - **RBCs:** The most distinctive but **not pathognomonic** feature is the presence of **spherocytes** in the peripheral smear. These spherocytes appear as abnormally small, dark-staining (hyperchromic) red cells without any central pallor (normally central 1/3rd is pale). Polychromatophilia in the peripheral smear is because of high reticulocyte count (reticulocytosis).
    Note: Spherocytosis can also be found in autoimmune hemolytic anemias.
  - **WBCs:** Total leukocyte count (TLC) is increased.
  - **Platelets:** Normal in number.
- **Reticulocyte:** Increased.

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Bone Marrow

- **Cellularity: Markedly hypercellular**
- **Erythropoiesis: Erythroid hyperplasia** with an increased number of normoblasts.
- **Myelopoiesis:** It is normal.
- **Megakaryopoiesis:** It is normal.

Biochemical Findings

- Serum bilirubin is mildly increased and mainly of unconjugated type.
- Urine urobilinogen is increased.
- Serum haptoglobin is decreased.

Osmotic Fragility Test

Osmotic fragility is increased in HS and there is shift of the curve to the right as shown in Fig. 46.1 (refer details in Chapter 46).

HEREDITARY ELLIPTOCYTOSIS

Hereditary elliptocytosis (HE) is a group of inherited disorders (usually autosomal dominant) involving the cytoskeleton of the red cells. Most of the patients are asymptomatic. Peripheral blood smear shows numerous elliptocytes (Fig. 6.4).

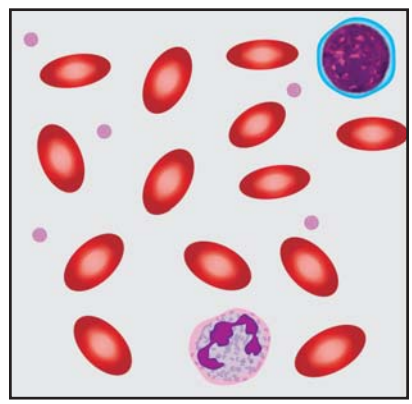


Fig. 6.4: Peripheral smear with numerous elliptocytes

SUMMARY

- Hereditary disorders causing hemolytic anemia include defect in the red cell membrane or red cell enzymes.
- Hereditary spherocytosis (HS) is a group of disorders due to defect in the red cell membrane, usually due to spectrin or ankyrin deficiency.
- The three key clinical manifestations are anemia, jaundice and splenomegaly.
- Spleen sequesters the abnormal spherocytes and causes extravascular hemolysis.
- The peripheral smear shows spherocytes and the osmotic fragility test is abnormal.

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SELF-ASSESSMENT EXERCISES

I. Essay

Discuss the etiology and diagnosis of hereditary spherocytosis.

II. Short Notes

1. Laboratory findings in hereditary spherocytosis.
2. Mention two conditions where you get spherocytes in the peripheral smear and write briefly on hereditary spherocytosis.
3. Etiopathogenesis and mechanism of hemolysis in hereditary spherocytosis.

III. Multiple Choice Questions

1. In hereditray spherocytosis mutations can occur in any of the following EXCEPT
A. Ankyrin
B. Band 3
C. Spectrin
D. Glycophorin C

2. The laboratory findings observed in hereditary spherocytosis is
A. Increased MCV
B. Decreased MCHC
C. Decreased erythropoiesis
D. Increased osmotic fragility

3. The peripheral blood in hereditary spherocytosis will show following features EXCEPT
A. High reticulocyte count
B. RBC without central pallor
C. Increased MCV
D. Increased MCHC

4. Aplastic crisis in hereditary spherocytosis is triggered by
A. Parvovirus infection
B. EBV infection
C. Infection by HBV
D. Measles

5. The important confirmatory test for a diagnosis of hereditary spherocytosis is
A. Ham's test
B. Osmotic fragility test
C. Autohemolysis test
D. Electrophoresis

6. The hematological disorder benefited by splenectomy is
A. Thalassemia
B. Hereditary spherocytosis
C. Sickle cell anemia
D. Pernicious anemia

ANSWERS

1. D **2.** D **3.** C **4.** A **5.** B **6.** B

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CHAPTER 7 Thalassemia Syndromes

CHAPTER OUTLINE

- Introduction
- β-Thalassemia
- α-Thalassemia

INTRODUCTION

Normally, equal quantities of α and β-globin chains are produced during the synthesis of normal adult hemoglobin (HbA). Thalassemia syndrome develops when one of these chains are reduced or absent and results in excessive production of the other chain.

The term *thalassemia* is derived from the Greek word "thalassic" that means "great sea" (high incidence in patients of Mediterranean descent) and anemia.

Definition

The **thalassemia syndrome** is a heterogeneous group of inherited disorders which result from a genetic abnormality of globin production. There is decreased or absence of synthesis of either α or β-globin chain of adult hemoglobin, HbA ($\alpha_2 \beta_2$).

- Thalassemia syndromes are endemic in Mediterranean region, Asia, Middle East, tropical Africa (and their American descendants) and Indian subcontinent.

Classification

Thalassemia syndromes are mainly classified into two types depending on the defective globin chain.

- **β-Thalassemia syndromes:** These are characterized by the impaired synthesis of β-chains of globin. β-thalassemia syndrome is the commonest and is prevalent all over the world including India.
- **α-Thalassemia syndromes:** These syndromes are characterized by reduced synthesis of α-chains of globin. These are seen mainly in South East Asian countries and India.

Clinical presentations: They vary from totally asymptomatic carriers to severe anemia. Clinical and hematological features of thalassemia syndromes are due to globin chain imbalance.

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- **Diminished synthesis of one globin chain** leading to **microcytic hypochromic** red blood cells.
- Relative **excess** (unequal production and accumulation of the free chain) of **other globin chain** (α in β-thalassemia and β in α-thalassemia). This leads to **ineffective erythropoiesis** and hemolytic anemia.

β-THALASSEMIA

β-Thalassemia is a hereditary disorder characterized by **diminished synthesis** of structurally **normal β-globin chains**, associated with unimpaired synthesis of α-chains. The clinical severity of the anemia varies due to heterogeneity in the causative mutations.

Molecular Pathology

β-globin genes are located on chromosome 11. At the molecular level, many **different types of mutations in β-globin gene** has been identified in β-thalassemia. These mutations may result in partial or complete deletion of a gene, or affect a single nucleotide base. For example, a single nucleotide base substitution by different base, results in a point mutation (most predominantly found). Others mutations include insertion or deletion.

Depending on whether β-globin chains are produced or not, the causative mutations are mainly categorized into two.

- **β^0 mutations**, characterized by **total absence of β-globin synthesis.**
- **β^+ mutations**, characterized by **markedly reduced or diminished (but detectable) β-globin synthesis.**

Clinical and Genetic Classification of β-thalassemia

They are broadly classified into 3 types (Table 7.1) depending on the severity of the anemia, which in turn depends on the genetic defect (β^+or β^0).

Table 7.1: Clinical and genetic classification of β-thalassemias

Clinical syndromes	*Genotype*	*Clinical features*
β-thalassemia major	Homozygous (β^0/β^0,β^+/β^+) or double heterozygous (β^0/β^+)	Severe form, severe anemia and transfusion dependent High level of HbF in the blood
β-thalassemia intermedia	Variable (β^0/β^+, β^+/β^+, β^0/β, β^+/β)	Moderately severe and not transfusion dependent
β-thalassemia minor/ β-thalassemia trait	Heterozygous (β^0/β, β^+/β)	Mild anemia and asymptomatic

β^0 = Total absence of β-globin synthesis; β^+ = Markedly reduced or diminished β-globin synthesis; β-normal β-globin synthesis

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Pathophysiology of β-thalassemia (Fig. 7.1)

A. Consequence of Defective or Absent β-chains

- **Anemia:** It is produced by following mechanisms:
  - **Diminished synthesis of HbA:** Lack of production of hemoglobin (HbA) causes microcytic hypochromic red cells (not enough Hb to fill up the red cells).
  - **Ineffective erythropoiesis:** The term ineffective erythropoiesis is used to describe condition in which the **bone marrow** is attempting to produce cells, but **not able to release viable cells into the circulation.** β-thalassemia major is associated with either **absence** (β^0) **or reduced synthesis of β-chains** (β^+). Since, α-chain synthesis is normal, these unstable **free α-chains accumulate, precipitate** and form intracellular, insoluble inclusions in the developing normoblasts and red cells. **Normoblasts** with such inclusions fail to mature and **undergo apoptosis** in the marrow (intramedullary) and result in **ineffective erythropoiesis.**

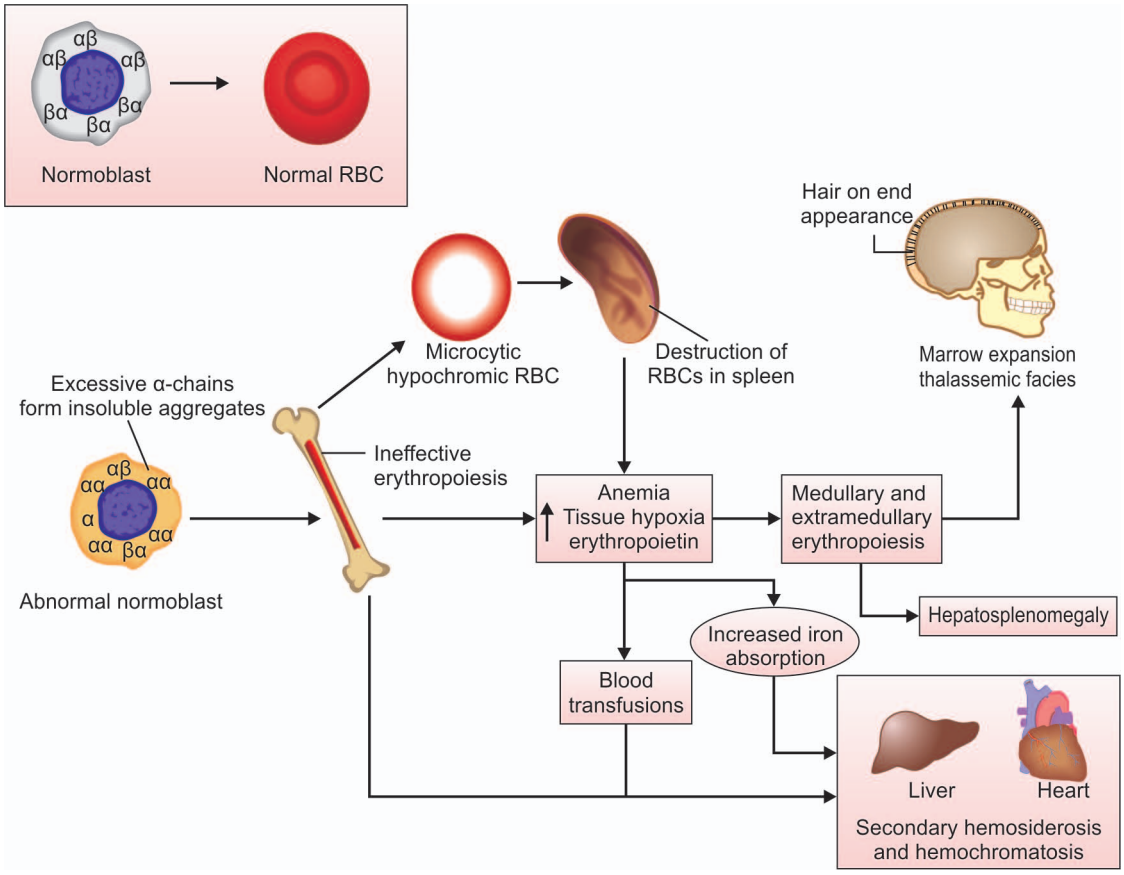


Fig. 7.1: Pathogenesis of β-thalassemia major and its consequences

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  - **Extravascular hemolysis:** The α-chain inclusions cause red cell membrane damage. The free α-chain inclusion-bearing red cells derived from abnormal precursors (normoblasts) escaping intramedullary apoptosis are **destroyed in the spleen** resulting in extravascular hemolysis and contribute to hemolytic anemia.
- **Synthesis of fetal hemoglobin (Hb F):** Even though **synthesis of γ-chains** disappear in infants after 6 months of age, its production may **continue in thalassemia**. They combine with α-chains resulting in **markedly increased level of Hb F($\alpha_2 \gamma_2$)** the level of which varies from 20 to 90%.

B. Consequences of Ineffective Erythropoiesis

- **Changes in bone marrow:** Severe hemolytic anemia stimulates erythropoietin (EPO) production by kidney. EPO acts on bone marrow and causes **marked erythroid hyperplasia.**
- **Changes in bone:** The expanding erythropoietic marrow erodes the bony cortex and stimulates new bone formation on the outer aspect. This is seen especially in the **skull vault, maxilla and facial bones. Skull X-ray** demonstrates classical **hair on end ("crew-cut") appearance** due to new bone formation (Fig. 7.2). A typical facies known as **thalassemic facies** (Fig. 7.3) develops, with prominent forehead (also called frontal bossing), cheekbones and upper jaw. X-rays of metatarsals, metacarpals and phalanges demonstrate a mosaic pattern because of trabeculations.
- **Extramedullary hematopoiesis:** As bone marrow cannot compensate for the anemia, extramedullary hematopoietic foci develop in **liver and spleen** and consequent hepatosplenomegaly.
- **Cachexia:** Severe anemia causes a hypoxic state in most of the tissues and the metabolically active erythroid progenitors compete for the essential nutrients with these tissues. In untreated patients this may lead to cachexia.

C. Iron Overload and its Consequences

- **Causes of iron overload:**
  - **Increased absorption of dietary iron from duodenum:** Hepcidin synthesized in the liver is the central regulator of iron homeostasis. Hepcidin controls iron absorption and storage. **Ineffective erythropoiesis suppresses the hepcidin** levels in the blood. Low levels of hepcidin result in **increased iron absorption.**
  - **Hemolysis**
  - **Repeated transfusions** (which is the usual mode of treatment).
- **Consequences:** The progressive iron overload causes **hemosiderosis** and **secondary hemochromatosis.** Excessive iron deposition often damages parenchyma of organs, most notable in **heart** (important cause of death), **liver** and **pancreas.**

β-Thalassemia Major

β-thalassemia major (Mediterranean or Cooley's anemia) is the homozygous form of β^0/β^0 or β^+/β^+ or double heterozygous β^0/β^+ (Table 7.1). It is most common in Mediterranean countries, parts of Africa and Southeast Asia.

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Clinical Features

- Infants with thalassemia major are well at birth but develop moderate to severe anemia 6-9 months after birth, when hemoglobin synthesis switches from HbF to HbA.
- Clinical course is short if not treated (mainly by multiple blood transfusions). Untreated/untransfused children **fail to thrive** (growth retardation) and die early within 4-5 years of age from the effects of anemia.
- Those who survive longer, marrow hypercellularity causes expansion of marrow and its widening give the **classical X-ray changes** (Fig. 7.2). Bones of the face are enlarged and distorted giving a characteristic **thalassemic facies** (Fig. 7.3).



Fig. 7.2: X-ray appearance of skull in β-thalassemia showing hair-on-end appearance (*Courtesy:* Dr Nuthan Kamath)



Fig. 7.3: Appearance of typical thalassemic facies (*Courtesy:* Dr Nuthan Kamath)

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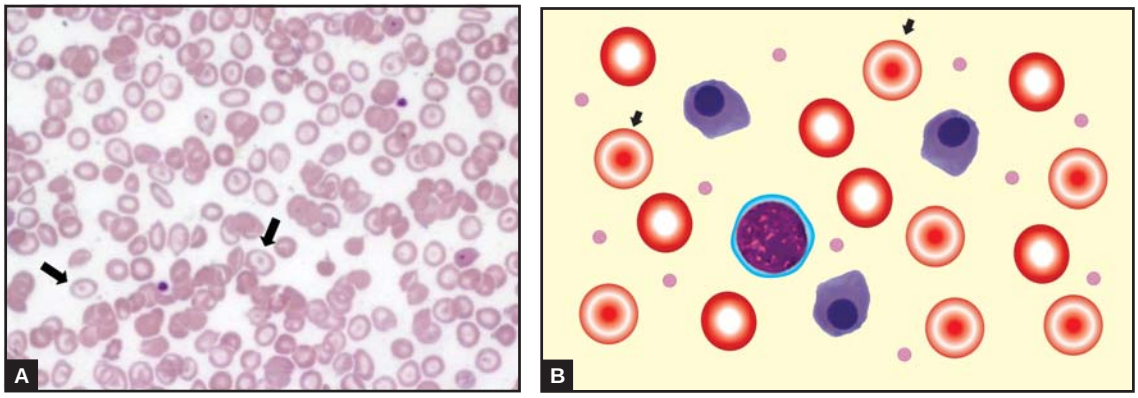
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- **Splenomegaly**: Spleen enlarges up to 1500 gm because of work hyperplasia and extramedullary hematopoiesis.
- **Liver and lymph nodes** also may show **extramedullary hematopoiesis.**
- Although blood transfusions improve the anemia but iron overload will lead to **hemosiderosis and secondary hemochromatosis**, which mostly damages organs like heart, liver and pancreas. Bone marrow transplantation is curative.

Laboratory Findings

Peripheral blood

- **Hemoglobin**: Anemia is moderate to severe and hemoglobin is in the range of 3-8 gm/dL in patients who have not been given transfusion.
- **RBC count:** It is increased (in contrast to iron deficiency anemia).
- **Hematocrit:** It is decreased and ranges from 8 to 23%.
- **Red cell indices**:
  - **MCV:** It is markedly diminished in the range of 45-70 fl.
  - **MCHC:** It is decreased to 22 to 30 g/dL.
  - **MCH:** It is diminished to 20-28 pg.
  - **RDW:** However the red cell distribution width (RDW) is **within normal limits** (in contrast to iron deficiency anemia where it is increased).
- **Reticulocyte count:** It is increased and in the range of 5 to 15%. But, because of the ineffective erythropoiesis, it is lower than expected for the severity of anemia.
- **Peripheral smear**
  - **RBCs:**
    - They show marked ***microcytic hypochromic anemia*** with moderate to marked variation in size (anisocytosis) and shape (poikilocytosis).
    - Many **target cells** (hemoglobin collects in the center of RBCs) are present (Figs 7.4A and B). Basophilic stippling (see Fig. 42.5) is also common. Aggregated a chains are removed by the spleen and not visible in the smear.



Figs 7.4A and B: Peripheral blood smear in β-thalassemia showing target cell (arrows) and three nucleated red cells (in Figure B)

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  - **Nucleated red cell precursors** (normoblasts) in variable numbers are demonstrable. They are due to "stress" erythropoiesis and also released from extramedullary hematopoietic sites.
- **WBCs:** There is leukocytosis with mild left shift.
- **Platelets:** Normal in number.

Bone marrow

- **Cellularity:** Bone marrow is **markedly hypercellular.**
- **M:E ratio:** It is reversed to 1:1 to 1:5 depending upon the degree of erythroid hyperplasia.
- **Erythropoiesis:** It is normoblastic with marked erythroid hyperplasia.
- **Myelopoiesis:** Normal.
- **Megakaryopoiesis:** Normal.
- **Bone marrow iron:** It is **markedly increased** because of increased dietary absorption and hemolysis.

Biochemical findings

- Bilirubin is increased and is mainly of unconjugated type.
- Urine urobilinogen is increased making urine high colored.
- Serum haptoglobin is markedly reduced.
- Serum iron status:
  - Serum iron, serum ferritin and transferrin saturation are markedly increased.
  - **Total iron binding capacity (TIBC) is reduced.**

Special tests

- **Hb F** level is **increased**. The levels are higher in β^0 thalassemia than in β^+ thalassemia (Table 7.2).
- **Hemoglobin electrophoresis**
  - Demonstrates bands of both Hb A and Hb F in β^+ thalassemia (β^+/β^+ or β^0/β^+ genotypes).
  - In β^o thalassemia (β^0/β^0 genotype), since no β-chains are formed there is no Hb A. Major hemoglobin is Hb F with normal or low Hb A_2.
- High Performance chromatography (HPLC) is a chromatographic technique used as confirmatory test.
- Prenatal diagnosis is possible by molecular analysis of DNA.

Table 7.2: Hemoglobin F and A_2 percentage in thalassemia syndromes

Type	*Hb F*	*Hb A_2*
β-Thalassemia major (homozygous)	30-90%	<3.5%
β-Thalassemia intermedia (double heterozygous)	10-30%	<3.5%
β-Thalassemia minor/trait (heterozygous)	0-5%	3.6-8%

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Thalassemia Intermedia

Thalassemia intermedia is a clinical entity in which patients have a clinical spectrum intermediate between thalassemia trait and thalassemia major. Patients are anemic, but are not transfusion dependent.

Thalassemia Minor

β-thalassemia minor is more common than β-thalassemia major. Most patients are heterozygous for thalassemic gene. They are usually asymptomatic and anemia is mild if present. They are usually detected when peripheral blood smear is examined for some other disease.

Clinical Features

Patients are asymptomatic without any splenomegaly or any other positive finding.

Laboratory Findings in Thalassemia Minor

- ***Peripheral blood:*** The peripheral blood smear usually shows red cell changes like microcytosis, hypochromia, basophilic stippling and target cells.
- ***Bone marrow:*** It shows mild erythroid hyperplasia.
- ***Hemoglobin electrophoresis:*** On hemoglobin electrophoresis an increase in HbA_2 ($\alpha_2\delta_2$) to 4 to 8% of the total hemoglobin (normal 2.5 ± 0.3%) is observed. HbF levels can be normal or slightly increased.

Differential diagnosis: β-thalassemia trait should be differentiated from the microcytic hypochromic anemia of iron deficiency. Measurement of serum iron, total iron-binding capacity and serum ferritin are helpful for differential diagnosis. Hemoglobin electrophoresis and HPLC are very helpful confirmatory test for β-thalassemia trait.

α-THALASSEMIA

- The α-thalassemia inherited disorders are characterized by **reduced or absent synthesis of α-globin chains.**
- Normally each chromosome 16 has a pair of α-globin genes. Thus each cell has four globin genes coding for four functional α-globin chains. α-globin chains are required for three normal variants of hemoglobin namely HbF (α_2, γ_2), HbA (α_2, β_2) and HbA_2 ($\alpha_2\,\delta_2$).

Molecular Pathology

In α-thalassemia, **gene deletion of α-gene is the most common cause** of reduced α-chain synthesis.

- Anemia is due to both, lack of adequate hemoglobin and the effect of excess unpaired non-α-chains (β, γ, δ).
- Synthesis of different non-α-chains varies in different phases of development.

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- Since, the β and γ-chain synthesis are normal, these free chains accumulate and precipitate. They from intracellular, insoluble inclusions in the developing normoblasts and red cells (Fig. 7.5). These inclusions cause red cell membrane damage and are responsible for hemolysis. Because of more solubility of free β and γ-chains in comparison to α-chains, hemolysis and ineffective erythropoiesis are less severe than in β-thalassemia.

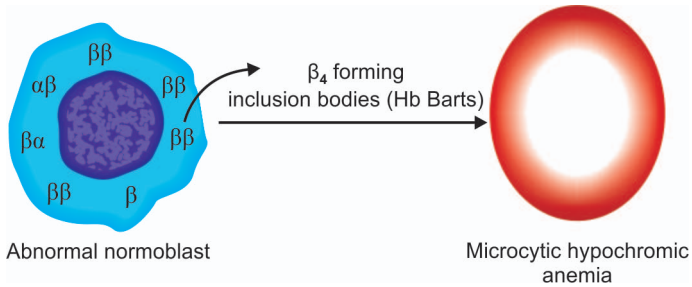


Fig. 7.5: Abnormal normoblast in α-thalassemia

Clinical Syndromes

The clinical syndromes and severity of α-thalassemia varies greatly depending on the number of α-globin genes deleted or affected (Table 7.3) which may range from 1 to 4. Each of the four α-globin genes normally contributes 25% of the total α-globin chains.

Table 7.3: Clinical syndromes associated with α-thalassemia disorders

Clinical syndrome	*No of α-globin deleted*	*Clinicopathological features*
Silent carrier state	1	Asymptomatic
α-Thalassemia trait	2	Usually asymptomatic. Normal hemoglobin level or minimal anemia
Hemoglobin H disease.	3	Moderate microcytic hypochromic anemia
Hydrops fetalis (Hb Barts)	4	Severe form, fatal and usually results in intrauterine death

Silent Carrier State

When only **single α-globin** gene is deleted, there is no significant decrease in α-globin chain synthesis. So, these individuals are asymptomatic and the peripheral smear may show only microcytosis.

α-Thalassemia Trait

It results from the deletion of **two α-globin genes** from a single chromosome (-/- α/α) or the deletion of one α-globin gene from each of the two chromosomes (α/- α/-). The patients are without any physical abnormality and peripheral blood smear may show microcytosis with minimal anemia if any.

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Hemoglobin H Disease

It is due to deletion of **three α-globin** genes. As there is only one normal α-globin gene, the synthesis of α-chains is markedly decreased. HbH has a very high affinity for oxygen and therefore cannot deliver oxygen to tissues. This results in tissue hypoxia disproportionate to hemoglobin level. Reticulocytes show tiny, uniform Hb H inclusions.

Hydrops Fetalis

It is the most severe form of α-thalassemia in which all **four α-globin** genes are deleted. The excess γ-globin chains form γ_4 tetramers (hemoglobin Barts) which have a high affinity for oxygen and therefore cannot deliver oxygen to tissues. Fetal distress develops in the third trimester of pregnancy. The fetus dies within the uterus and shows severe pallor, generalized edema and massive hepatosplenomegaly (hydrops fetalis), similar to that of hemolytic disease of the newborn.

SUMMARY

- Thalassemia syndrome is a heterogeneous group of disorders in which one or more globin chain synthesis is either absent or reduced.
- Thalassemia syndrome results in microcytic hypochromic anemia due to decreased production of hemoglobin with erythroid hyperplasia of the bone marrow.
- The imbalance of globin synthesis causes an excess of the normally produced globin chain. This results in damage to red blood cells or their precursors and hemolysis.
- Thalassemia syndrome is mainly of two types, α and β.
- β-thalassemia may clinically manifest as thalassemia major, intermedia and minor with decreasing severity. β-thalassemia major is a severe disorder and transfusion-dependent hemolytic anemia. Radiological appearance (hair on end appearance) on the skull X-ray and thalassemic facies are due to expansion of bone marrow due to erythroid hyperplasia.
- α-thalassemia is due to absence or reduced production of α-globin chain. In α-thalassemia, the degree of anemia depends on the number of α chains (out of normal 4) that is deleted. Hydrops fetalis is characterized by deletion of all α genes and is incompatible with life.

SELF-ASSESSMENT EXERCISES

I. Short Notes

1. Beta thalassemia
2. Molecular pathogenesis of beta thalassemia
3. Laboratory findings in beta thalassemia
4. List the causes and laboratory diagnosis of microcytic hypochromic anemia
5. Differences between iron deficiency anemia and thalassemia minor
6. Differences between iron deficiency anemia and thalassemia major
7. Differences between thalassemia major and minor

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II. Multiple Choice Questions

1. **The thalassemia syndrome is due to decreased or absence of synthesis of**
   A. β-globin chains
   B. α-globin chains
   C. γ-globin chains
   D. Either α or β-globin chain
2. **β-Thalassemia syndromes is characterized by impaired synthesis of**
   A. β-globin chain
   B. α-globin chain
   C. Both α and β-globin chain
   D. Either α or β-globin chain
3. **The molecular defect NOT found in thalassemia is**
   A. Single-nucleotide (or point) mutations
   B. Base substitutions
   C. Insertion or deletion mutations
   D. Truncation
4. **In β-thalassemia major, all the following laboratory findings may be found EXCEPT**
   A. Diminished HbA synthesis
   B. Decreased total iron binding capacity
   C. Presence of target cells
   D. Increased red cell distribution width
5. **Crew cut appearance on skull X-ray is found in**
   A. Thalassemia major
   B. Pernicious anemia
   C. Multiple myeloma
   D. Burkitt lymphoma
6. **The following is NOT a feature of β-thalassemia minor**
   A. Marked increase in HbA_2
   B. Increased HbF
   C. Microcytic hypochromic RBC
   D. Severe anemia
7. **HbA_2 concentration in β-thalassemia minor is usually**
   A. Less than 1%
   B. 1-2.5%
   C. 2.5-3.5%
   D. More than 3.5%
8. **At the molecular level, the most common cause of alpha thalassemia is**
   A. Amino acid substitutions in the α-chain
   B. Deletion of α-genes
   C. Excess α-chain production
   D. Deletion of β-genes
9. **This is an α-thalassemic disorder**
   A. HbH disease
   B. Cooley's anemia
   C. Pernicious anemia
   D. Sideroblastic anemia

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10. The γ_4 tetramers are known as
A. Heinz bodies
B. Pappenheimer bodies
C. Hb Barts
D. Cabot rings

11. Hydrops fetalis is fatal because
A. HbH cannot bind to oxygen
B. The excess β-globin chains form insoluble precipitates
C. Microcytic red cells gets trapped in the placental blood vessels
D. Hb Barts cannot deliver oxygen to tissues

12. The following is NOT seen in β-thalassemia major
A. Microcytic hypochromic anemia
B. Erythroid hyperplasia
C. Raised HbF
D. Raised total iron binding capacity (TIBC)

13. Hemoglobin electrophoresis in β-thalassemia major will reveal
A. Decreased HbA
B. Decreased HbA_2
C. Decreased HbF
D. Increased HbH

ANSWERS

1. D	**2.** A	**3.** D	**4.** D	**5.** A	**6.** D	**7.** D
8. B	**9.** A	**10.** C	**11.** D	**12.** D	**13.** A	

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CHAPTER 8 Sickle Cell Disease

CHAPTER OUTLINE

- Introduction
- Sickle Cell Anemia
- Sickle Cell Trait
- Other Sickling Syndromes
- Other Hemoglobinopathies

INTRODUCTION

Sickle cell disease (SCD) is an important group of hereditary disorders of hemoglobin characterized by production of defective hemoglobin called sickle hemoglobin (HbS). HbS imparts sickle shape to red cells on low oxygen tension or deoxygenation. The term sickle cell disease includes all entities associated with sickling of the red blood cells.

Classification of Sickle Cell Disease

Sickle Cell Anemia (SS)

- It is a homozygous state in which both the β-globin chains are abnormal or defective, with one defective gene each from both the parents.
- Clinically, these patients manifest early in life, since HbS constitutes more than 70% of hemoglobin with no HbA being produced.

Sickle Cell Trait (AS)

- It is a heterozygous state in which one gene from one parent is defective for HbS (abnormal) while the other gene is for HbA (normal).
- These patients have both Hb A and Hb S in their red cells with absence of significant clinical features. Amount of HbS varies from 25 to 40% and may remain undetected.

Other Sickling Syndromes (Compound Heterozygous)

- If both the β-globin chains have different abnormalities, (e.g. Hb SC, Hb S-β-thalassemia) they are termed as **compound heterozygous**.
  A family structure of sickle cell anemia and trait is shown in Fig. 8.1.

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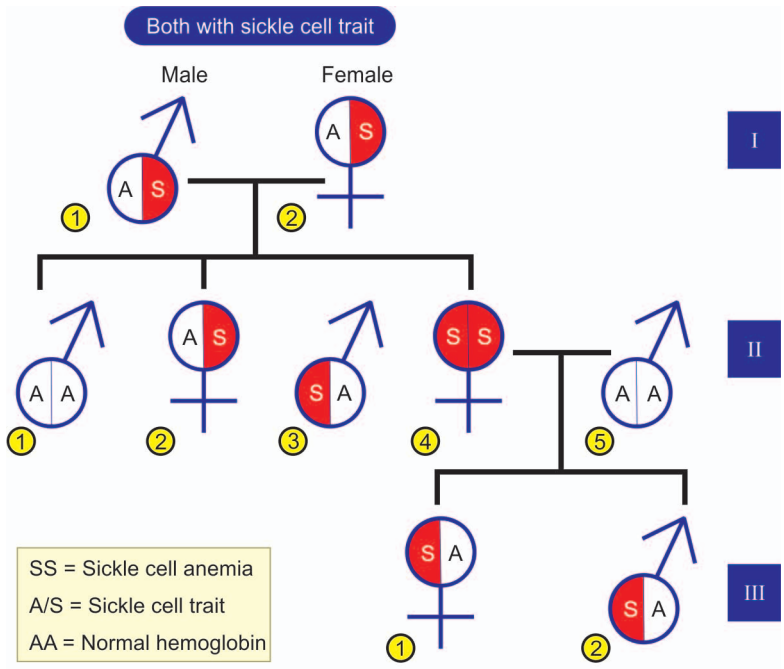


Fig. 8.1: A family structure with both parents (I) having sickle cell trait and with 25% possibility of sickle cell anemia (SS) in the offspring (II). Each child of sickle cell anemia patient (III) will have sickle cell trait (SA) if the other parent is normal (AA)

SICKLE CELL ANEMIA

Definition

Sickle cell anemias is a homozygous state (SS) caused by a mutation in the β-globin gene. This hemolytic anemia is characterized by vaso-occlusive disease, widespread acute and chronic organ damage and a reduced lifespan.

Sickle cell anemia patients have mainly HbS (more than 70% of hemoglobin) in their red cells. It has been suggested that **HbS provides protection against falciparum malaria.**

Etiopathogenesis

- Sickle cell anemia is caused by production of abnormal hemoglobin called sickle hemoglobin (HbS).
- In HbS, there is an adenine (A) to thymidine (T) substitution (GAG→GTG) in codon 6 of the β-globin gene.
- This point mutation results in **replacement of the normal glutamic acid residue by valine** and alters the solubility or stability of the hemoglobin. The resultant HbS is defined by the structural formula $\alpha_2\beta_2^S$ ($\alpha_2\beta_2^{6Glu\text{-}Val}$), and is responsible for the characteristics of the disease.

It is discussed under following subheadings namely: (1) molecular basis of sickling, (2) factors affecting sickling, (3) mechanism of red cell damage and (4) pathogenesis of the microvascular occlusions.

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Molecular Basis of Sickling (Fig. 8.2)

- In a state of low O_2 tension or deoxygenation**, HbS molecules undergo aggregation and polymerization**. Initially, aggregates of HbS formed results in conversion of the red cell cytosol from a freely flowing liquid to a viscous gel.

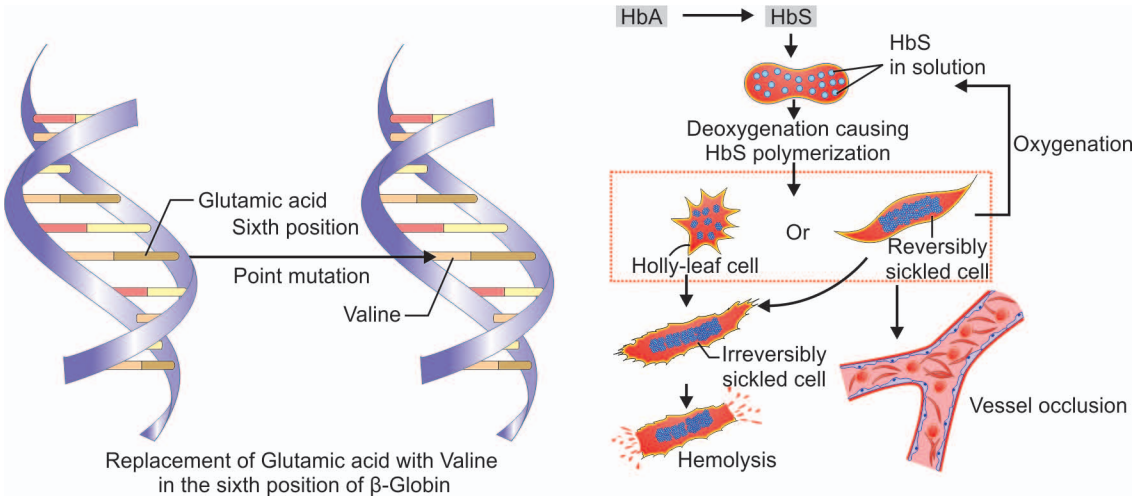


Fig. 8.2: Pathogenesis of sickle cell anemia

- If deoxygenation continues, the aggregated HbS molecules form long needle-like fibers or pseudo-crystalline structures known as tactoids within red cells. These pseudo-crystalline structures grow in length beyond the diameter of red cell. This distorts the shape of red cell to an elongated **sickle or crescent** moon or holly-leaf shape.
- Sickle cells do not alter their shape, which occurs normally during their passage in the splenic circulation. This predisposes to stasis and vascular occlusion.
- When the oxygen tension returns to normal, the Hb S polymers dissolve and the sickle cell reverts to its normal shape.
- Repeated episodes of sickling causes red cell membrane damage and cells become **irreversibly sickled (ISC)**, retaining their abnormal shape even when fully oxygenated.
- The precipitation of HbS fibers also causes oxidant damage, not only in irreversibly sickled cells but also in normal-appearing cells.

Factors Affecting Sickling (Table 8.1)

- **Amount of other hemoglobins**
  - **Adult hemoglobin (HbA): HbA** interferes with HbS polymerization and **decreases sickling.**

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Table 8.1: Factors affecting sickling

Factors	Favors sickling	Hinders sickling
Amount of other hemoglobins	-	**HbA**
	-	**HbF**
	HbC	-
Transit time in microvasculature	**Slowing of blood stream**	-
MCHC	**Increased MCHC**	**Decreased MCHC**
Intracellular pH.	**Decreased pH**	-
Other factors	**Temperature above 37°C**	-
	Infections	-

  - In **sickle cell trait** (**heterozygous),** approximately 40% of the hemoglobin is HbS and 60% is HbA. Such individuals remain as asymptomatic carriers and their red cells sickle only with severe hypoxia.
  - In contrast, **homozygous HbS** individuals with no HbA have full-blown sickle cell anemia.
- **Fetal hemoglobin (HbF):** It prevents the polymerization of HbS and decreases sickling. So, infants up to 6 months do not manifest symptoms, when the HbF level begins to fall close to adult levels.
- **Hemoglobin C: It favors sickling** by inhibiting ion exchange across the RBC membrane and thus individuals with HbS and HbC (designated HbSC disease) have increased sickling.

- **Transit time in microvasculature:** Normally, transit time in microvasculature is very brief preventing aggregation of deoxygenated HbS.
  - **Slowing of blood flow** results in exposure of red cells to hypoxia for more duration which results in **sickling.** The slowing of blood flow is observed in inflammation.
- **Mean corpuscular hemoglobin concentration (MCHC).**
  - Higher HbS concentration and intracellular dehydration result in increased MCHC, which facilitate sickling.
  - Conditions which decrease the MCHC reduce the severity of the disease. Hydrating the cells decreases sickling.
- **Intracellular pH:** A decrease in pH increases the tendency for sickling e.g. acidosis.
- **Other factors:** Temperature above 37°C and infections promote sickling.

Mechanism of Red Cell Damage

Several mechanisms are involved in which sickling causes damage to red cells.

- **Growing HbS beyond membrane:** As the HbS polymers grow, they project through the cell membrane covered only by the lipid bilayer.
- **Dehydration:** Repeated episodes of sickling causes increasing dehydration of red cells which become dense and rigid. Finally, the most severely damaged cells reach an irreversible, nondeformable end-stage known as **irreversibly sickled cells (ISC)**. These ISC cannot revert back to normal and retain the sickle shape even if fully oxygenated later. This irreversibility is because of permanent deformity of the submembrane cytoskeleton.

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- **Percentage of ISC:** The degree of the hemolysis correlates with the percentage of irreversibly sickled cells. The damaged ISCs are removed by erythrophagocytosis in the spleen, liver or bone marrow. Thus hemolysis is mainly extravascular, but mechanically fragile sickled cells can also cause minor degree of intravascular hemolysis.
- **Impaired cation homeostasis:** The structural changes in the membrane causes the influx of Ca^{+}ions, which activate an ion channel resulting in the efflux of K^{+} and H_2O.

Pathogenesis of the Microvascular Occlusions

The most ominous clinical features of sickle cell anemias are due to occlusion of microvasculature. Even though the exact pathogenesis is uncertain, several possibilities have been thought of.

- **Deformability:** Sickle cells have difficulty in changing their shape when they pass through the microvasculature. These rigid cells may aggregate and block the small blood vessels.
- **Factors that slow the blood flow:** Occlusion may be dependent upon red cell membrane cytoskeletal damage and other factors (e.g. inflammation) that tend to slow or arrest the movement of red cells through microvascular beds.
- **Higher expression of adhesion molecules**:
  - Sickle cells express higher levels of adhesion molecules and become abnormally sticky.
  - Chemical mediators released from leukocytes during inflammation may up-regulate the expression of adhesion molecules on endothelial cells. These mediators favor adhesion and stagnation of these sticky sickle cells in inflamed microvasculature. The resulting low oxygen tension favors sickling and microvascular obstruction.
- **Inactivation of nitric oxide:** Nitric oxide (NO) is a potent vasodilator and inhibitor of platelet aggregation. Lysed sickle cells liberate free hemoglobin which binds and inactivates NO. Inactivation of NO narrows the vessels and favors platelet aggregation, resulting in microvascular stasis and sickling.

Once started, this vicious cycle consisting of sickling, obstruction and hypoxia continues. There is no relation between the number of irreversibly sickled cells and microvascular occlusions.

Clinical Features (Fig. 8.3)

The clinical presentation of sickle cell disease changes as life advances**.** Presence of HbF in the first 6 months of life has a protective role. Symptoms appear after 6 months of age as the HbF disappears. Infants and children present with acute problems like severe infection, acute chest syndrome, splenic sequestration and stroke. Chronic hypoxia in children is responsible for generalized impairment of growth and development. Adults manifest with chronic organ damage.

The cardinal clinical features are due to: chronic hemolytic anemia, crises (recurrent painful episodes), infections and chronic organ damage.

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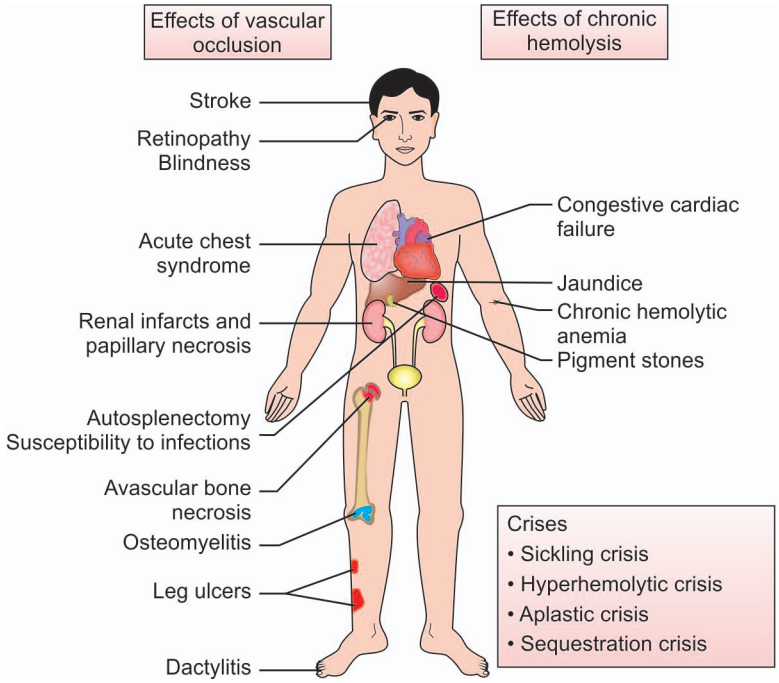


Fig. 8.3: Various effects of vascular occlusion and hemolysis in sickle cell anemia

A. *Chronic Hemolytic Anemia*

Patients suffer from lifelong hemolysis, but most patients have moderate anemia. Hemolysis is mainly extravascular, but sickled red cells being mechanically fragile can cause intravascular hemolysis also. Chronic hemolytic anemia causes increased levels of unconjugated (indirect) bilirubin, which predisposes to development of pigmented bilirubin gallstones. Cholelithiasis may lead to cholecystitis.

B. *Crises*

Any new syndrome or episode that develops rapidly in sickle cell anemia is termed crises. The protracted course of sickle cell anemia is frequently exacerbated by a variety of crises. Four types of crises are encountered. These are:

1. *Sickling crisis (vaso-occlusive/pain/painful/infarctive crisis)*

The most common and the hallmark of sickle cell disease is **vaso-occlusive crisis**. Blockage of microcirculation by sickled red cells causes hypoxic injury and infarction. Clinically it presents with acute, severe pain in the affected region (severe abdominal, thoracic, muscle or bone pain). This type of crises may be precipitated by fever, infection and dehydration (conditions which favor sickling) or may be without any predisposing cause. It commonly involves bones, lungs, liver and spleen.

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- **Bone:** In children, bone involvement may resemble acute osteomyelitis. They manifest as the hand-foot syndrome, dactylitis of the bones of the hands or feet or both.
- **Lung:** It presents with fever, cough, chest pain and pulmonary infiltrate which is known as *acute chest syndrome (dangerous).* These are sometimes initiated by a simple lung infection.
- **Spleen:** Acute abdominal pain lasting for 4-5 days, may be the presenting symptom caused by infarcts of abdominal viscera due to vaso-occlusive crises lasting 4-5 days. Recurrent splenic infarction leads to complete involution of spleen with impaired or absent splenic function (autosplenectomy).

2. *Hyper hemolytic crisis*

This is rare type and presents with marked increase in hemolysis with a sudden lowering of hemoglobin and reticulocytosis.

3. *Aplastic crisis*

A transient cessation of bone marrow erythropoiesis may develop due to an acute infection of erythroid progenitor cells by parvovirus B19. Reticulocytes disappear from the peripheral blood causing a sudden and rapid worsening of anemia. Recovery starts within 5 to 10 days.

4. *Sequestration crisis*

Usually occurs in children with chronically enlarged but normal functioning spleen. Sudden trapping of blood in spleen or liver causes rapid enlargement of the organ with resultant drop in hematocrit and hypovolemic shock.

Other crises encountered rarely are hypoplastic crisis and megaloblastic crisis (due to inadequate folate).

C. *Infections*

Children with sickle cell anemia are susceptible to acute infections with encapsulated organisms. Common infections are *pneumonia due to pneumococcus, meningitis* due to *S. pneumonia* and *osteomyelitis* due to *Salmonella.* Increased susceptibility to infections is because of:

1. *Hypofunction of spleen*
   - In children, it is due to congestion and poor blood flow.
   - In adults, it is due to multiple infarcts and resultant autosplenectomy.
2. *Defects in the alternative complement pathway*

   This defect impairs opsonization of encapsulated bacteria such as *pneumococci* and *Haemophilus influenzae*.

Septicemia and meningitis are the most common causes of death in children. Increased frequency of osteomyelitis is because of repeated bone infarcts which act as a nidus for infection.

D. *Chronic Organ Damage*

Chronic hypoxic damage may be particularly seen in the spleen, bones, kidneys, heart, lungs, brain and skin.

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- **Spleen**
  - *Children* after 6 months of life present with splenomegaly up to 500 gm.
  - After 5-6 years of age, there is gradual reduction in the size of spleen as vaso-occlusion of splenic artery branches causes *multiple infarcts* which get fibrosed and spleen is reduced in size.
  - In severely affected adults only a small nubbin of fibrous tissue is left and its function becomes abnormal. Gradual loss of splenic function secondary to infarcts is called **autosplenectomy**.
- **Bone:** Osteomyelitis, particularly with *Salmonella typhimurium*, due to impaired splenic function and avascular necrosis of the femoral head is often seen.
- **Kidney:** Sickling commonly occurs in the renal medulla and is responsible for renal infarcts and papillary necrosis. Patient may develop hyposthenuria (inability to concentrate urine), which increases the risk of dehydration and its consequences.
- **Genital organ:** Male patients may develop priapism.
- **Heart:** Anemia results in chronic demand resulting in increased cardiac output which causes cardiomegaly and congestive heart failure. Coronary occlusion may cause myocardial ischemia.
- **Brain:** In the central nervous system, hypoxia can provoke seizures or stroke.
- **Retina:** Retinal hemorrhage and detachment, proliferative retinopathy and blindness may develop.
- **Extremities:** Skin ulcers over the lower extremities, especially near the ankles, due to obstruction of dermal capillaries.

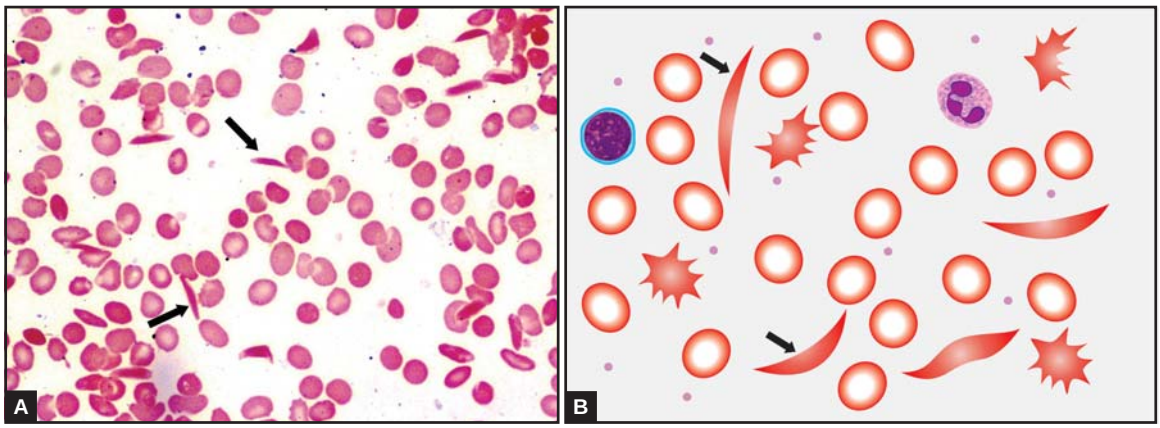
Laboratory Findings in Sickle Cell Anemia

Peripheral Blood

- **Hemoglobin:** It is decreased and usually in the range of 5-10 gm/dL.
- **Hematocrit (PCV) :** Decreased and usually in the range of 18 to 30%.
- **Reticulocytosis**: Increased reticulocyte count and usually in the range of 5 to 20% is observed. Higher reticulocyte count is observed when hemolysis is of severe degree.
- **ESR:** Low because sickle cells do not form rouleaux.
- **Peripheral smear**
  - **RBCs:**
    - **Red blood cells** are normocytic normochromic to mildly hypochromic.
    - There is moderate to severe degree of anisopoikilocytosis.
    - The characteristic cell which is seen in the smear is the **sickle cell.** These appear as long, curved cells with pointed ends (Figs 8.4A and B). Smear may also show **target cells (**due to red cell dehydration) **and ovalocyte**s. Number of sickle cells is variable.
    - There is polychromatophilia due to reticulocytosis.
    - Few RBCs show Howell- Jolly bodies (small nuclear remnants), which is due to hypofunction of spleen resulting from autosplenectomy.
    - Few nucleated RBCs are also observed.

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Figs 8.4A and B: Peripheral blood smear with sickle cells (arrows)

- **WBCs:** Total count is mildly increased with shift to left as in most other hemolytic anemias.
- **Platelets:** Platelet count is mildly increased.

Bone Marrow

- **Cellularity:** Bone marrow is **hypercellular.**
- **Erythropoiesis:**
  - The bone marrow hypercellularity is because of a compensatory normoblastic erythroid hyperplasia. However, chronic hemolysis can result in secondary folate deficiency and bone marrow may show megaloblastic type of hyperplasia.
  - Bone marrow hyperplasia expands the marrow causing resorption of bone and secondary new bone formation. In severe cases, these changes result in prominent cheek bones and skull bone which resemble a **crew-cut** in roentgenograms.
- **Myelopoiesis:** Within normal limits.
- **Megakaryopoiesis:** Within normal limits.
- **Iron stores:** These are usually increased.

Extramedullary hematopoiesis can also develop as a compensatory mechanism.

Serum Findings

- **Iron status:** There is increase in serum iron, serum ferritin and transferrin saturation.
- **Serum haptoglobin:** It is decreased.
- **Serum bilirubin:** The increased hemolysis can cause hyperbilirubinemia and consequently pigment gallstones.

Urine Urobilinogen

It is increased.

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Diagnostic/confirmatory Tests

- **Sickling test**: Sickling is induced by adding a reducing (oxygen-consuming) agent like 2% sodium metabisulphite or sodium dithionite to blood sample.

  Method
  - Prepare a 2% sodium metabisulphite solution.
  - Take a clean glass slide.
  - Take 1 drop of 2% sodium metabisulphite solution.
  - Add ½ drop of patient's blood on the glass slide.
  - Mix it properly and place a cover slip on the slide.
  - Seal the edges of the cover slip with paraffin wax or nail polish.
  - Examine the slide under the microscope.
    - Immediately and
    - After 15 minutes and ½ hour or upto 24 hours for any sickling.

  Result: Red cells with HbS show ***sickled*** (Fig. 8.5) ***and holly leaf*** appearance.

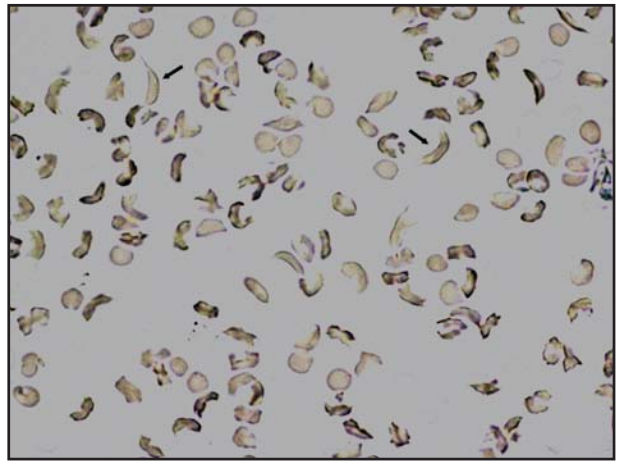


Fig. 8.5: Sickling test. Sickled red cells (arrows) induced by reducing agent (2% sodium metabisulfite)

- **Hemoglobin electrophoresis:** HbS is a slow moving hemoglobin compared to Hb A and Hb F.
- **Estimation of Hb F:** Hb F in homozygous state constitutes about 10-30% of hemoglobin. Hence, it is necessary to carry out Hb F estimation (alkali denaturation method).
- **HPLC:** High-performance liquid chromatography is useful for confirmation of diagnosis.
- Prenatal diagnosis can be done by analysis of fetal DNA obtained by amniocentesis or chorionic villous biopsy.

SICKLE CELL TRAIT

Sickle cell trait is the heterozygous state (AS) for the hemoglobin S mutation and shows both HbA and HbS (HbAS).

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Pathogenesis

In patients with sickle cell trait, the hemoglobin A in their red cells prevents hemoglobin S polymerization and hence, normally they do not undergo sickling. However, RBCs may sickle under extreme conditions (e.g. flight at high altitude in unpressurized aircraft, deep sea diving).

Clinical Features

Sickle cell trait individuals are usually asymptomatic. They have a normal growth and development, life span and life expectancy.

Laboratory Findings

Peripheral Blood

- **Hemoglobin:** It is either normal or mildly decreased and ranges from 11-13 gm/dL.
- **Peripheral smear**
  - **RBCs:** They show normocytic normochromic picture with very few target cells and mild degree of anisopoikilocytosis.
  - **WBCs:** Within normal limits.
  - **Platelets:** Adequate in number.

Bone Marrow

The bone marrow is hypercellular because of a compensatory normoblastic erythroid hyperplasia.

Diagnostic Tests

- *Hb electrophoresis:* It demonstrates two bands of Hb S and Hb A.
- *Sickling test:* Sickling test using 2% sodium metabisulphite or sodium dithionite is positive.
- *HPLC:* High-performance liquid chromatography is useful for confirmation of diagnosis.

OTHER SICKLING SYNDROMES

These are characterized by the presence of one HbS gene and other abnormal hemoglobin gene (compound heterozygous). They include:

Hemoglobin Sβ Thalassemia

In this the patient inherits S gene and β thalassemia gene from each parent. The clinical manifestations vary according to severity of β gene defect. Hb S β^0 clinically resembles sickle cell anemia and is associated with reduced MCV in addition to sickle cells.

Hemoglobin SE Disease

It presents with mild microcytic hypochromic anemia. Electrophoresis demonstrates HbS and E.

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Hemoglobin SD Disease

This simulates but is less severe than sickle cell anemia. On routine (alkaline) electrophoresis, the pattern is indistinguishable from sickle cell anemia because Hb S and Hb D cannot be separated. Citrate agar electrophoresis at pH 6 will separate Hb S and Hb D.

OTHER HEMOGLOBINOPATHIES

Other hemoglobinopathies are uncommon and manifest as heterozygous or homozygous state. In the homozygous state, they produce only mild chronic hemolysis with splenomegaly. These hemoglobinopathies includes:

Hemoglobin D Punjab

It is also known as Hb D Los Angeles, is seen in India especially in Punjab and Gujarat. It results from point mutations in the β globin chain gene at position 121.

Hemoglobin D Trait

- It is a heterozygous state and patients are asymptomatic.
- Peripheral smear may show few target cells.
- Hemoglobin electrophoresis demonstrates HbA and HbD

Hemoglobin D Disease

- It is a homozygous state and manifests with mild hemolytic anemia (Hb ranges from 7 to 10 gm/dL) and splenomegaly.
- Peripheral smear may show polychromasia and few target cells.
- Hemoglobin electrophoresis (citrate agar at pH6) demonstrates HbD (90 to 95%) HbA and HbF.

Hemoglobin E

It results from substitution of glutamic acid by lysine in the 26th position of β globin chain gene. In India it is seen in Bihar, West Bengal and Assam.

Hemoglobin E Trait

It is a heterozygous state and patients are asymptomatic.

Hemoglobin E Disease

- It is a homozygous state and manifest mild anemia and splenomegaly.
- Peripheral smear may show microcytosis and many target cells.
- Hemoglobin electrophoresis demonstrates HbE (93 to 98%).

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Hemoglobin C

It results from substitution of glutamic acid by lysine in the 6th position of β globin chain gene.

Hemoglobin C Trait

- It is a heterozygous state and patients are clinically normal.
- Peripheral smear may show target cells (5 to 20%).

Hemoglobin C Disease

- It is a homozygous state and manifest mild anemia and splenomegaly.
- Peripheral smear may show numerous target cells (upto 95%).
- Hemoglobin electrophoresis demonstrates HbC (75 to 90%) and HbF. HbA is absent.

The gene for HbC is often inherited with that for HbS. HbS-C disease behaves as a mild sickle disease with a tendency for venous thrombosis.

SUMMARY

- Sickle cell disease is a hereditary disorder of hemoglobin characterized by production of defective sickle hemoglobin.
- Sickle cell anemia is homozygous state due to substitution of valine for glutamic acid at the sixth position of the β-globin chain. HbS present in red blood cells, when polymerized, gives rise to sickle shaped red cells.
- Sickle cells can cause mainly four types of crises namely vaso-occlusive, aplastic, hemolytic and sequestration crisis.
- Sickle cell trait is a heterozygous state and usually asymptomatic.

SELF-ASSESSMENT EXERCISES

I. Essay

Describe the etiopathogenesis and peripheral blood findings of sickle cell anemia.

II. Short Notes

1. Sickle cell anemia
2. Pathogenesis of sickle cell disease
3. Factors affecting sickling
4. Crises in sickle cell anemia
5. Differences between sickle cell anemia and trait
6. Laboratory findings in sickle cell anemia

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III. Multiple Choice Questions

1. **In sickle cell anemia replacement of the glutamic acid residue by a valine occurs at the 6th position of**
   A. α-globin chain
   B. β-globin chain
   C. γ-globin chain
   D. δ-globin chain
2. **Factor which does NOT favor sickling in sickle cell anemia is**
   A. Infection
   B. Slowing of blood stream
   C. HbF
   D. Increased MCHC
3. **"Autosplenectomy" may develop in patients with**
   A. Hereditary spherocytosis
   B. Aplastic anemia
   C. Sickle cell anemia
   D. Thalassemia
4. **The hemolytic anemia that does NOT produce splenomegaly is**
   A. Hereditary spherocytosis
   B. Hereditary elliptocytosis
   C. Sickle cell anemia
   D. Thalassemia
5. **Children with sickle cell anemia are susceptible to**
   A. Osteomyelitis due to *Salmonella*
   B. Tuberculosis due to atypical mycobacterium
   C. Syphilis by treponema pallidum
   D. Lepromatous leprosy due to mycobacterium leprae
6. **In sickling test, sickling is induced by**
   A. 5% sodium chloride
   B. 2% sodium metabisulphite
   C. 3% sodium citrate
   D. 5% sodium trithionate
7. **The agent which can cause aplastic crisis in sickle cell anemia is**
   A. *Salmonella*
   B. *S. pneumonia*
   C. Parvovirus B19
   D. Epstein Barr virus
8. **Vaso-occlusive crisis leading to infarcts occurs in**
   A. Hereditary spherocytosis
   B. α-thalassemia
   C. β-thalassemia
   D. Sickle cell anemia

ANSWERS

1. B **2.** C **3.** C **4.** C **5.** A **6.** B **7.** C **8.** D

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Chapter 9 Hemolytic Anemias due to Red Cell Enzyme Deficiencies

Chapter Outline

- Metabolic Pathways in Red Blood Cells
- Glucose-6-phosphate Dehydrogenase Deficiency
- Pyruvate Kinase Deficiency

METABOLIC PATHWAYS IN RED BLOOD CELLS

Energy for the red cells is available primarily through glycolysis by two major pathways. The two pathways of glycolysis are:

- **Embden-Meyerhof pathway:** It is the major source of energy (ATP production), which provides high energy phosphate for maintenance of (i) membrane lipids and (ii) sodium and potassium.
- **Hexose monophosphate (HMP) shunt pathway:** Even though only 10% of glucose is catabolized by this pathway, it is important for prevention of oxidative damage to red cells. Glucose-6-phosphate dehydrogenase (G6PD) is an enzyme involved in HMP shunt and is one of the free radical–scavenging systems.

Abnormalities in the form of deficiency or impairment of any of the enzymes of these pathways will affect the red cell metabolism.

Hemolytic Disease due to Red Cell Enzyme Defects

Red cell enzyme defects reduce the ability of red cells to protect themselves against oxidative injuries and are susceptible to hemolysis. The hemolysis associated with defects in glycolysis is known as congenital **nonspherocytic hemolytic anemia** because the RBC morphology is minimally altered. Red cell enzyme deficiencies encountered are:

- *Hexose monophosphate shunt enzyme deficiencies:* G6PD deficiency, glutathione synthetase deficiency.
- *Glycolytic (Embden-Meyerhof pathway) enzyme deficiencies:* Pyruvate kinase deficiency, hexokinase deficiency.

GLUCOSE-6-PHOSPHATE DEHYDROGENASE DEFICIENCY

RBC is susceptible to injury by oxidants of both exogenous and endogenous origin. It has to protect itself from this oxidative damage by free radical injury. This is achieved by reduced glutathione (GSH).

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Role of G6PD (Fig. 9.1)

- High concentration of reduced glutathione (GSH) in the normal RBCs protects them against oxidant injury by catalyzing the breakdown of compounds such as H_2O_2 to H_2O.
- Reduced glutathione is generated by the hexose monophosphate shunt. Adequate concentration of reduced glutathione (GSH) is maintained by reduced form of nicotinamide adenine dinucleotide (NADPH) which converts oxidized glutathione (GSSG) to reduced glutathione. The housekeeping enzyme, G6PD reduces nicotinamide adenine dinucleotide phosphate (NADP) to NADPH while oxidizing glucose-6-phosphate (Fig. 9.1).

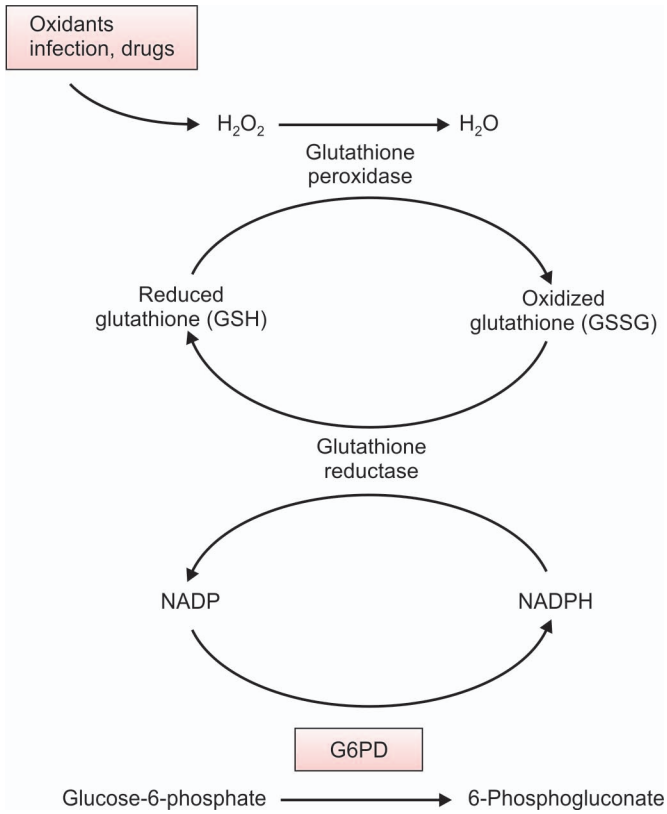


Fig. 9.1: Role of G6PD against injury by oxidants

Sequence of Events in G6PD Deficiency

In G6PD deficiency, oxidants can cause both intravascular and extravascular hemolysis, but it is mainly of extravascular type. The sequence of events is:

- In G6PD deficiency, there is **impaired glutathione in reduced state** and red cells are susceptible to oxidative damage.
- Hemolysis develops when RBCs are exposed to conditions that generate **oxidant stress**. These oxidants may be produced by the following conditions:

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- Infections: They generate oxygen-derived free radicals from activated leukocytes. These infections include viral hepatitis, pneumonia and typhoid fever.
- Drugs or chemical exposure: These include: antimalarials (e.g. primaquine and chloroquine), sulfonamides, nitrofurantoins and others.
- Certain foods: Fava beans generate oxidants when metabolized and can cause hemolysis.
- Unknown: Uncommonly, neonatal jaundice or a chronic low-grade hemolytic anemia may be presented without any triggering event.

- G6PD deficient **red cells accumulate H_2O_2** which damages erythrocyte membrane and hemoglobin resulting in hemolytic anemia.
- **Hemolysis liberates hemoglobin.**
- **Oxidation of hemoglobin** through oxidants **forms methemoglobin** in which Fe^{2+} ions are converted to ferric (Fe^{3+}) ions. Methemoglobin cannot transport oxygen, and since it is unstable, it forms membrane-bound precipitates in the cytoplasm known as **Heinz bodies** (Fig. 9.2). Heinz bodies are seen as dark inclusions within red cells with supravital stains (as in the reticulocyte preparation-Chapter 43).
- When these red cells with Heinz bodies pass through the spleen, macrophages pluck these Heinz bodies. This membrane damage gives red cells an abnormal shape, appearing to have a bite taken out of them. These are called as **"bite cells"** and appear in the peripheral blood (Fig. 9.2). Other less severely damaged cells revert to a spherocytic shape due to loss of membrane surface area.
- Both bite cells and spherocytes are highly susceptible to trapping and removal via **erythrophagocytosis in the spleen.**

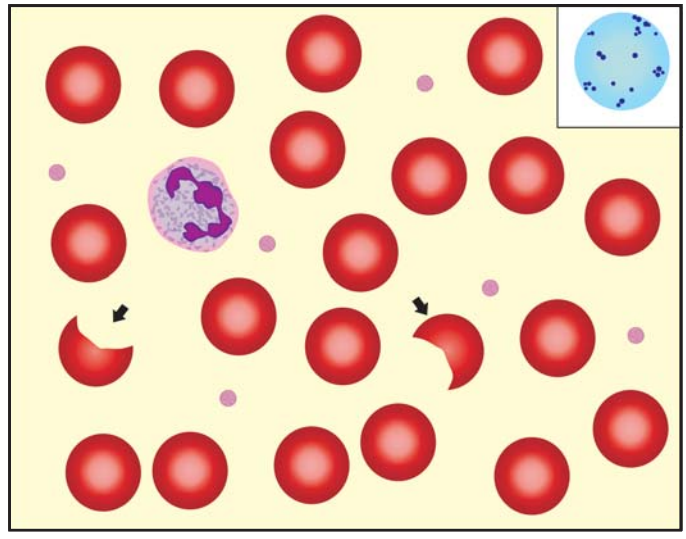


Fig. 9.2: Peripheral blood smear in G6PD deficiency with "bite cells" (arrows). Inset shows Heinz bodies (supravital stain)

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Table 9.1: Variants of G6PD

WHO classification-class	*Severity of G6PD deficiency*	*Hemolysis*	*G6PD variants*
I	Severely deficient	Chronic hemolysis	
II	Severely deficient (<10%)	Acute episodes	G6PD-Mediterranean
III	Moderately to mildly deficient (10 to 60%)	Acute episodes	G6PD-A^{-}
IV	Normal (60-150%)	Absent	G6PD-B, G6PD-A^{+}
V	Increased	Absent	

Variants of G6PD (Table 9.1): More than four hundred G6PD genetic variants are known, but majority are harmless. These variants differ in their activity, stability and electrophoretic mobility. These are classified into five classes depending on the degree of deficiency and hemolysis (Table 9.1). These variants are given geographic names (Mediterranean) or other types (A^{+}, A^{-} or B). Normal G6PD is generally designated as G6PD-B. Normally, the young red cells have highest G6PD activity which decreases as the red cells become old. This predisposes the older red cells to hemolysis more than younger ones. G6PD A^{-} or G6PD Mediterranean which have moderate to severe enzyme deficiency results in inability to protect against oxidative injury as red cells become old.

- *G6PD A^{-}:* The minus sign indicates absence of enzyme activity. Hemolysis develops when exposed to oxidant drugs or infections. The hemolysis is mild to moderate and is limited to the older RBCs.
- *G6PD Mediterranean:* It is prevalent in the middle east and there is marked decrease of enzyme stability and activity.

Prevalence and Malaria

G6PD deficiency has a protective effect against plasmodium falciparum malaria, since the ribose products required for the nucleic acid synthesis of malarial parasite are not available.

Genetics of G6PD

G6PD deficiency is an X-linked recessive trait and its full expression is seen only in males. Rarely, homozygous females can suffer from the disease. Heterozygous females will be asymptomatic carriers because they have two populations of red cells; normal cells and G6PD deficient cells. This dual population of cells in heterozygous females is because of the phenomenon of X-chromosome inactivation (Lyon's hypothesis).

Clinical Presentation

G6PD deficiency manifests in several distinct clinical patterns. They are: (1) acute hemolytic anemia, (2) chronic hemolytic anemia, (3) neonatal hyperbilirubinemia and (4) favism.

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Acute Hemolytic Anemia

Most common presentation is acute hemolysis after exposure to oxidant stress like drugs and infections mentioned earlier. This type of presentation develops in male patients. The patients with **acute hemolytic episode present with:**

- Pallor due to sudden development of anemia.
- Passage of dark urine due to hemoglobinuria.
- Jaundice due to hemolysis.

Laboratory findings

Peripheral blood

- **Hemoglobin:** It is decreased. There is sudden fall to 6-10 gm/dL following hemolysis.
- **Reticulocyte count:** It increases to 20-25% and depends on the degree of hemolysis. The recovery phase following hemolysis is characterized by reticulocytosis.
- **Peripheral smear:**
  - **RBCs:** They show moderate degree of anisopoikilocytosis with polychromatophilia, microspherocytes and **bite cells** (Fig. 9.2).
  - **WBCs:** Show mild leukocytosis.
  - **Platelets:** Appear adequate.
- **Self-limited hemolysis**: Mainly the old red cells are hemolyzed, hence hemolysis is self limited. After about 10 days, hemoglobin levels gradually start rising and may return to normal level within 2 months.

Urine

Hemoglobinuria will be found during hemolysis and may last for about 1-6 days.

***Tests for* G6PD deficiency** are positive. G6PD levels should be assessed a few weeks after the acute hemolytic episode, because of increased reticulocytes which have high amount of G6PD and may mask the deficiency of G6PD. The diagnosis of G6PD deficiency is based on the generation of NADPH from NADP as detected by either quantitative spectrophotometric analysis or by a rapid fluorescent screening test.

Chronic Hemolytic Anemia (nonspherocytic)

In severe enzyme deficiency patient will have chronic hemolysis throughout life. This hemolytic anemia is mild and found in the absence of infection or known environmental triggers.

Neonatal Hyperbilirubinemia

Uncommonly, G6PD deficiency presents as neonatal jaundice. Infant may develop kernicterus.

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Favism

Hemolysis can also occur after ingestion of fava beans (favism), which generate oxidants when metabolized.

PYRUVATE KINASE DEFICIENCY

Pyruvate kinase (PK) deficiency is the most common enzyme deficiency in the Embden-Meyerhof pathway. It is an autosomal recessive disorder which causes congenital chronic hemolytic anemia. The peripheral blood film shows normocytic normochromic anemia and increased reticulocytes. It is associated with increased levels of erythrocyte 2,3-DPG because of the site of the metabolic block.

SUMMARY

- Hemolytic anemias due to red cell enzyme defects include G6PD deficiency and PK deficiency. These enzymes are involved in glycolytic pathways.
- Glucose-6-phosphate dehydrogenase indirectly through glutathione protects the red cells from oxidative damage.
- G6PD deficiency is an X-linked disorder that develops as acute hemolytic anemia (secondary to drug exposure or infection), or chronic nonspherocytic hemolytic anemia, or neonatal jaundice or favism.
- Quantitative assay of red blood cell G6PD enzyme activity will establish the diagnosis, but should not be performed immediately after the episode of hemolysis.
- Pyruvate kinase (PK) deficiency is an autosomal recessive disorder which causes congenital chronic hemolytic anemia.

SELF-ASSESSMENT EXERCISES

I. Short Notes

1. G6PD deficiency anemia
2. Heinz bodies and its demonstration

II. Multiple Choice Questions

1. Heinz bodies are found in

A. G6PD deficiency
B. Thalassemia
C. Sickle cell anemia
D. Iron deficiency anemia

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2. Removal of Heinz bodies present in abnormal RBCs by splenic macrophages gives rise to
A. Elliptocyte
B. Target cell
C. Bite cell
D. Schistocyte

3. The drug that can cause hemolytic anemia in a patient with G6PD deficiency is
A. Iron
B. Aspirin
C. Primaquine
D. Vitamin B_{12}

4. G6PD deficiency protects against
A. Malaria
B. Kala-azar
C. Atypical pneumonia
D. Development of acute hemolytic anemia

ANSWERS

1. A **2.** C **3.** C **4.** A

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Chapter 10
Immunohemolytic Anemia

Chapter Outline

- Definition
- Classification
- Alloimmune Hemolytic Anemia
- Autoimmune Hemolytic Anemia

DEFINITION

Immunohemolytic anemia is a group of anemias in which **premature red cell destruction** (hemolysis) is mediated **by antibodies** that bind to red cells.

- The antibodies may be either allo or autoantibodies. Autoantibodies are directed against self red cell surface antigens.
- The hemolysis is caused by extracorpuscular mechanisms.
- The site of hemolysis may be extravascular or intravascular.

CLASSIFICATION

Immunohemolytic anemia is classified depending on the characteristics of the responsible antibody (Table 10.1).

Table 10.1: Classification of immunohemolytic anemias

Alloimmune hemolytic anemia
- Hemolytic disease of the newborn
- Hemolytic transfusion reactions: Mismatched blood transfusion

Autoimmune hemolytic anemia

Warm antibody type (IgG antibodies active at 37°C)
- Primary (Idiopathic)
- Secondary
  - Autoimmune disorders (systemic lupus erythematosus)
  - Drugs
  - Lymphomas

Cold agglutinin type (IgM antibodies active at 4°C to 18°C)
- Acute
  - Mycoplasmal infection
  - Infectious mononucleosis
- Chronic
  - Idiopathic
  - Lymphomas

Cold hemolysin type (Donath-Landsteiner antibodies)

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ALLOIMMUNE HEMOLYTIC ANEMIA

In alloimmune hemolytic anemia, allo-antibodies are present either in the serum or bound to red cells.

Hemolytic Disease of the Newborn (HDN)

This is an immune hemolytic anemia which affects the fetus and newborn baby. It is due to maternal-fetal blood group incompatibility. HDN develops when the mother lacks an antigen that is expressed by the fetal red cells. HDN may be due to incompatibility of Rh type or ABO group.

Rh Hemolytic Disease of newborn (Fig. 10.1)

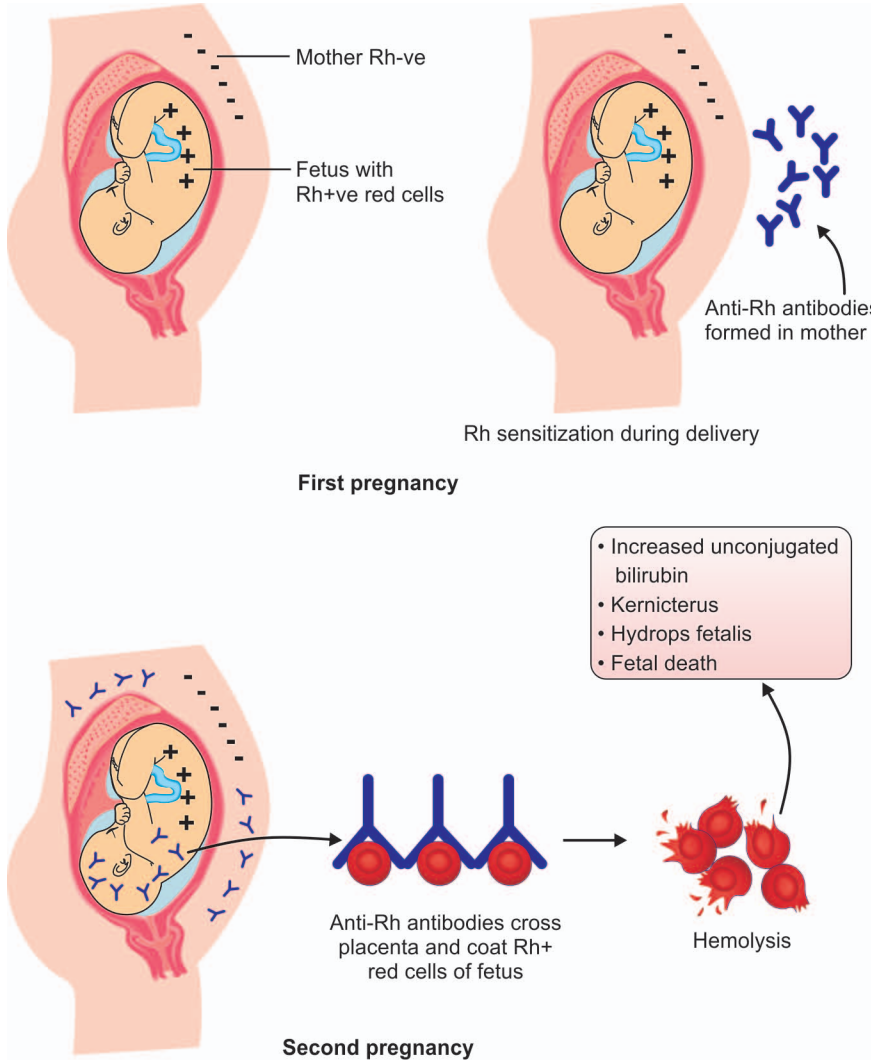


Fig. 10.1: Pathogenesis of Rh hemolytic disease of newborn

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Pathophysiology

- HDN occurs when an **Rh negative mother** is exposed to **Rh positive fetal red cells.**
- Passage of fetal red cells into the maternal circulation normally occurs only at the time of delivery or as a result of miscarriage. Thus, HDN **does not manifest in the first pregnancy.**
- These Rh positive cells sensitize an Rh negative mother and induce **formation of anti-Rh antibodies.**
- In subsequent pregnancies, Rh positive fetus stimulates antibody production in mother, initially IgM and subsequently IgG.
- **Antibodies cross placenta and coat the Rh positive fetal red cells** and cause immune **destruction of fetal red cells** in tissues of mononuclear phagocyte system, particularly spleen. This results in icterus of the newborn.
- The fetus compensates by increasing the red cell production in the bone marrow, liver and spleen causing hepatosplenomegaly.
- Anemia develops and causes cardiac dilatation, hypertrophy and subsequently cardiac failure.
- Hypoalbuminemia develops because of diminished liver function. The combination of reduced plasma oncotic pressure and increased hydrostatic pressure (due to cardiac failure) results in generalized edema and anasarca, resulting in **hydrops fetalis** (immune type). Hydrops fetalis is a fatal condition characterized by the accumulation of fluid causing generalized edema of the fetus during intrauterine growth. Hydrops fetalis may also be due to nonimmune cause like cardiovascular defects, chromosomal anomalies and fetal anemia (e.g. α-thalassemia in which there is deletion of all four α-globin genes).
- Hydrops fetalis occurs usually between 20 and 40 weeks of gestation and may eventually lead to intrauterine death.
- If fetus can compensate sufficiently (compensated hemolysis), the child may be born alive with some degree of HDN.

Clinicopathological features

- Most infants have no jaundice at birth. Severe disease is associated with a high titer of anti-D results in high levels of unconjugated bilirubin.
- In the fetus/infant, elevated levels of unconjugated bilirubin can pass the blood brain barrier and can get deposited in the central nervous system, especially the basal ganglia. This results in severe neurological damage, known as **kernicterus** (yellow coloration of cerebellum and basal ganglia due to bilirubin deposition). If not treated kernicterus leads to a severe encephalopathy and **death**.
- The disorder can be prevented by the prophylactic removal of fetal cells entering the maternal circulation before sensitization develops, by injecting anti-D into the Rh D negative mother.

Laboratory findings

Peripheral blood

- **Hemoglobin:** Decreased
- **Reticulocyte count:** Increased

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- **Peripheral smear:**
  - **RBCs:** Show normocytic normochromic anemia with numerous nucleated RBCs, polychromatophils and occasional spherocytes.
  - **WBCs:** They are within normal limits.
  - **Platelets:** Within normal limits.
- **Antiglobulin test (Coombs test):** Antibodies in the mother and baby are detected by indirect and direct Coombs test respectively.

Serum findings
- **Serum bilirubin:** Increased
- **Lactate hydrogenase (LDH):** Increased
- **Haptoglobin:** Decreased

ABO Hemolytic Disease of the Newborn

The fetus may be affected in the first pregnancy, where IgG antibody to A or B antigen on fetal red cells develops in a mother with O blood group. The IgG antibodies from maternal blood cross placenta and enter the fetal circulation. These anti-A or anti- B antibodies react with A and B antigenic determinants present in fetal fluids and tissues. This results in consumption of major portion of the maternal IgG and the small portion which is left combines with fetal red cells causing only mild hemolysis.

Hemolytic Transfusion Reactions (Chapter 60)

a. Immediate Hemolytic Transfusion Reaction

- An immediate massive hemolysis of the transfused blood develops when incompatible/mismatched blood is administered to a patient with preformed alloantibodies.
- In ABO mismatch, naturally occurring IgM type of antibodies present in the recipient's serum get activated at 37° C.
- These antibodies coat the donor red cells with complement and activation to C5-9 results in intravascular hemolysis.

b. Delayed Hemolytic Transfusion Reaction

- A transfusion reaction may occur up to 2 weeks after transfusion.
- This occurs in patient who has been alloimmmunized by a pregnancy or previous transfusion.

AUTOIMMUNE HEMOLYTIC ANEMIA

Autoimmune hemolytic anemia (AIHA) are a group of disorders in which antibodies against self-antigens on the RBC membrane cause premature destruction of RBCs. Presently the term AIHA is replaced by immunohemolytic anemia as few of these are not due to autoimmune mechanism. Anti-RBC antibodies can be divided into three general categories (Table 10.1).

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Interaction of the autoantibody with the red cell antigen is **dependent on the temperature, i.e. warm or cold type.**

Warm Antibody Type

- Warm antibody type is the most common type (50 to 70%) of immunohemolytic anemia.
- This can be further divided into **idiopathic** (primary) or **secondary** to drug exposure or predisposing disease.
- The antibodies are mainly of **IgG** type. The antibodies combine with red cell antigen at 37°C and hence known as **warm antibody**.
- These IgG-bound RBCs bind to Fc receptors on phagocytes (macrophages) which remove part of red cell membrane with attached antibody and hence known as **"partial" phagocytosis.** Progressive loss of membrane converts the red cells to **spherocytes.** As in hereditary spherocytosis, the spleen is the major site of removal and destruction of these spherocytes, resulting in moderate **splenomegaly.**

Etiology and Pathogenesis

Primary: The cause of autoantibody formation is largely unknown. In most of the cases, the antibodies are against the Rh blood group antigens.

- **Secondary (Table 10.1):** Immunological mechanism of is better understood with drug-induced hemolytic anemia. Three mechanisms of hemolysis are known.
  - **Hapten mechanism**: Some drugs (like penicillin and cephalosporins) cannot initiate immune reaction by themselves. But they act by hapten mechanism, where they bind to the proteins on the red cell membrane and become immunogenic. Antibodies of IgG type, directed against the cell-bound drug result in the destructive process (Fig. 10.2). The destruction of red cells occurs **mainly extravascularly** in the mononuclear phagocyte system (especially spleen). But destruction can **also occur intravascularly** after complement fixation.
  - **Immune complex mechanism (Fig. 10.3):** Drugs combine with plasma proteins to form a plasma protein drug complex. Antibodies are formed against this plasma protein drug complex which gets deposited on the RBC membrane. The antigen-antibody complex then binds with complement resulting in intravascular hemolysis.
  - **Autoantibody mechanism (Fig. 10.4):** Certain drugs (α-methyldopa) alter normal red cell antigens and initiate the production of autoantibodies directed against the altered red cell antigens (which is now not recognized as self), in particular the Rh blood group antigens. These autoantibodies bind the red cell membrane and result in extravascular hemolysis.

Clinical Features

AIHA can affect any age group and shows female preponderance. Onset is usually insidious, presenting with progressive pallor and jaundice. Splenomegaly is mild to moderate. History of drug intake must be excluded before diagnosing this condition.

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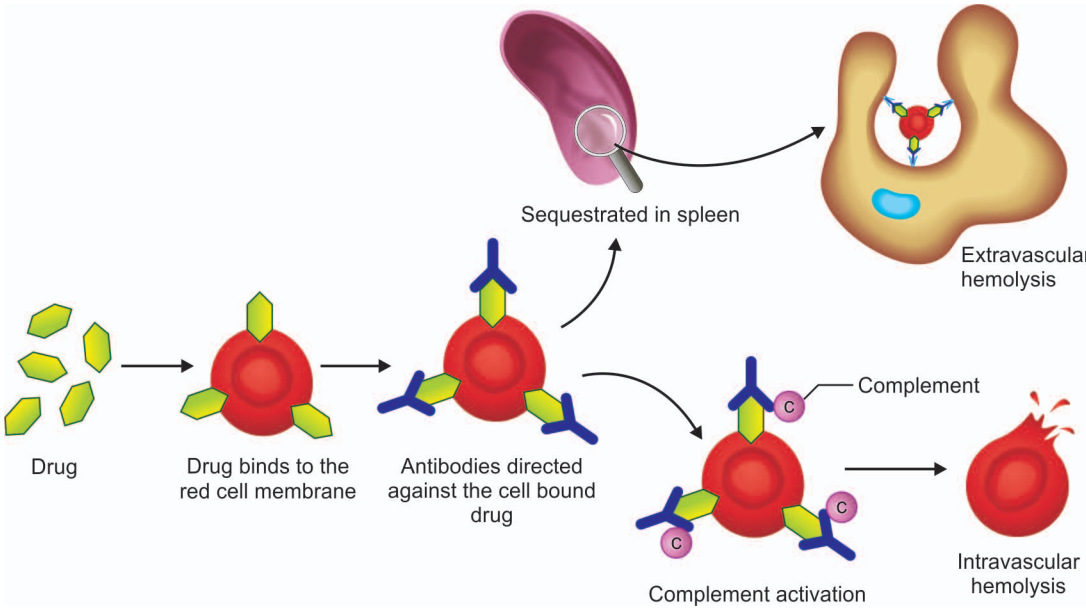


Fig. 10.2: Hapten mechanism of warm antibody type of AIHA

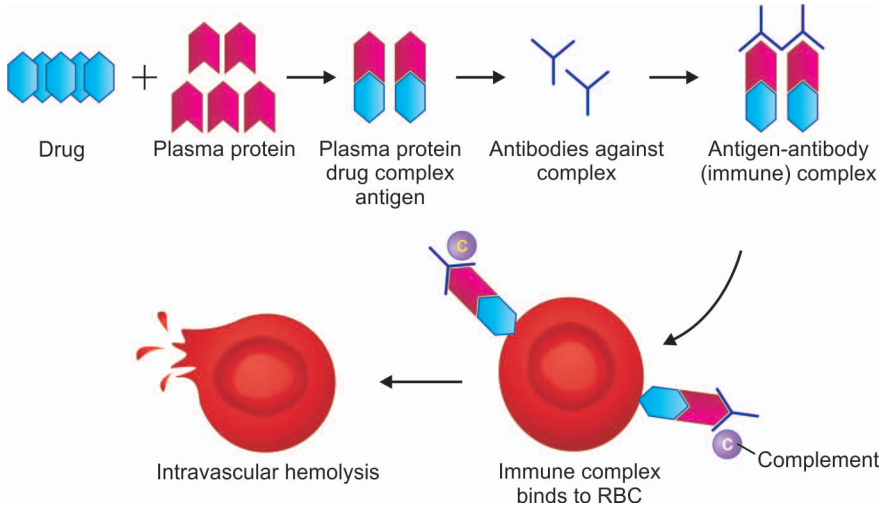


Fig. 10.3: Immune complex mechanism of warm antibody type of AIHA

Evans syndrome: This is a rare disease characterized by autoimmune thrombocytopenia and/or immune neutropenia with autoimmune hemolytic anemia. The underlying cause is not clearly defined.

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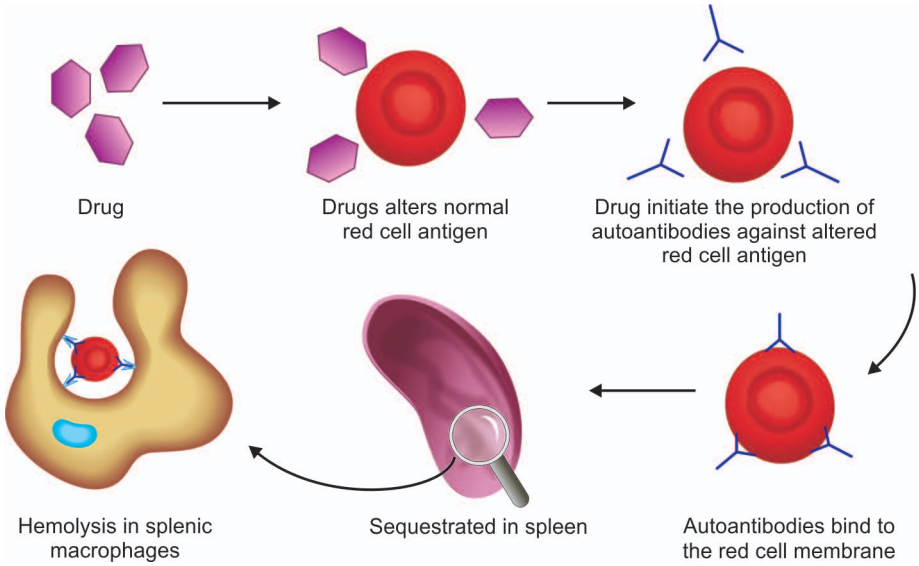


Fig. 10.4: Autoantibody mechanism of warm antibody type of AIHA

Laboratory Findings

Peripheral blood

- **Hemoglobin**: Hemoglobin is in the range of 5-9 gm/dL.
- **Hematocrit:** Decreased in the range of 14-28%.
- **Reticulocyte count**: Reticulocyte count is moderately increased in the range of 5-20% and higher counts are found during acute hemolytic episodes.
- **MCV:** Raised because of increased number of reticulocytes.
- **Peripheral smear**:
  - **RBCs:** Show normocytic normochromic anemia, polychromasia, nucleated RBCs and spherocytes.
  - **WBCs:** Monocytes may show erythrophagocytosis.
  - **Platelets:** Adequate
- **Bone marrow:** It shows erythroid hyperplasia.

Serum findings

- **Serum bilirubin**: Serum bilirubin (mainly of unconjugated type) is increased.
- **Serum LDH:** Increased because of hemolysis.
- Intravascular hemolysis results in hemoglobinemia, hemoglobinuria and hemosiderinuria.

Other tests

- **Direct antiglobulin test**: DAT (Coombs test) is usually positive in 90-95% cases (refer Chapter 59).
- **LE cell test:** Positive in SLE with secondary AIHA.

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Cold Agglutinin Type

- It is less common than the warm antibody type of immunohemolytic anemia and is caused by so-called ***cold agglutinins***.
- These antibodies bind to red cell antigens at **low temperatures** (4- 18°C), i.e. avidity of red cell to antibody increases as the temperature falls.
- The antibodies are of IgM type, fix complement and result in intravascular hemolysis or sensitized RBCs are lysed by RE cells of the liver and spleen (extravascular hemolysis).
- Clinically, it presents with cold induced acrocyanosis (i.e. blue color of the fingertips, toes, nose and ear lobes).
- There are **two clinical forms** of cold agglutinin immunohemolytic anemia.

  Acute form: Less common and always self-limited. It usually occurs during the recovery phase of certain infectious disorders, such as mycoplasma pneumonia, infectious mononucleosis, influenza and HIV-infections. It rarely causes clinically significant hemolysis.

  Chronic form: Most common and occurs in older persons. Many develop in association with B-cell lymphoid neoplasms (e.g. chronic lymphocytic leukemia, lymphoma) or as an idiopathic condition.

Laboratory Findings

Peripheral blood

- Hemoglobin, hematocrit, reticulocyte count findings are similar to that of warm antibody immunohemolytic anemia (page 102).
- **Peripheral smear:**
  - **RBCs:** Show normocytic normochromic anemia with polychromasia. The smear characteristically shows **red cell clumping or agglutination** along with spherocytes and nucleated RBCs.
  - **WBCs:** They are within normal limits.
  - **Platelets:** Adequate in number.

Serum findings

They are similar to warm antibody immunohemolytic anemia (page 102).

Other tests

Direct antiglobulin test (refer Chapter 59) is usually **positive.**

Cold Hemolysins Type (Donath-Landsteiner antibodies)

- These are autoantibodies directed against the P antigen system on red cells. They are responsible for a rare disorder known as ***paroxysmal cold hemoglobinuria***.
- The autoantibodies are of IgG type and bind to red cells at low temperatures and fix the complement. Since complement cascade functions more efficiently at 37°C, upon warming, complement gets activated resulting in intravascular hemolysis and hemoglobinuria.

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- They are usually transient and found mainly in children. It often follows viral infections and patients recover within a month.
- **Direct antiglobulin test** is usually **negative.**

SUMMARY

- Immunohemolytic anemias are classified as alloimmune and autoimmune hemolytic anemias.
- Alloimmune hemolytic anemia can be due to Rh or ABO incompatibility.
- Hemolytic disease of newborn mostly results from Rh incompatibility between Rh-ve mother and Rh +ve fetus. It is characterized by hemolytic anemia and hyperbilirubinemia.
- Autoimmune hemolytic anemias are caused by autoantibodies that bind to antigens on the red cell surface.
- The autoimmune hemolytic anemias are further subclassified as warm or cold reactive depending on the temperature at which the antibodies produce the effect.
- Warm reactive are common and due to IgG type antibodies with maximum reaction at 37° C.
- The drugs cause secondary autoimmune hemolytic anemia by different mechanisms like hapten, immune-complex or by autoantibodies.
- Cold agglutinin type of immunohemolytic anemias are caused by IgM anti-RBC autoantibodies which are active at temperature in the range of 4 to 18°C. It is triggered by exposure to cold.

SELF-ASSESSMENT EXERCISES

I. Short Notes

1. Hemolytic disease of newborn
2. Warm antibody immunohemolytic anemia

II. Multiple Choice Questions

1. **Hemolytic disease of the newborn develops with**
   A. Rh –ve fetus in an Rh +ve mother
   B. Rh +ve fetus in an Rh –ve mother
   C. Rh –ve fetus in an Rh –ve mother
   D. Rh +ve fetus in an Rh +ve mother
2. **The main type of antibody causing warm antibody type of immunohemolytic anemia is**
   A. IgA
   B. IgE
   C. IgM
   D. IgG

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3. **The peripheral smear in warm antibody immunohemolytic anemia shows RBCs which are**
   A. Acanthocytic
   B. Spherocytic
   C. Dacrocytic
   D. Elliptocytic
4. **Antibodies in warm antibody immunohemolytic anemia are usually directed against**
   A. I
   B. P
   C. Kidd
   D. Rh
5. **The disorder associated with secondary type of warm antibody immunohemolytic anemia is**
   A. ALL
   B. AML
   C. CML
   D. Lymphoma
6. **Yellowish discoloration of cerebellum and basal ganglia due to deposition of bilirubin is known as**
   A. Hydrops fetalis
   B. Jaundice
   C. Kernicterus
   D. Bilirubinemia
7. **The drug which causes warm antibody immunohemolytic anemia by hapten mechanism is**
   A. Penicillin
   B. Aldomet
   C. Quinidine
   D. Methyldopa
8. **Antibody in paroxysmal cold hemoglobinuria is directed against the blood group antigen**
   A. I
   B. P
   C. MNO
   D. Rh
9. **Donath Landsteiner antibodies are seen in**
   A. Warm antibody immunohemolytic anemia
   B. Paroxysmal cold hemoglobinuria
   C. Paroxysmal nocturnal hemoglobinuria
   D. Immune thrombocytopenic purpura

ANSWERS

1. B	**2.** D	**3.** B	**4.** D	**5.** D
6. C	**7.** A	**8.** B	**9.** B	

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CHAPTER 11 Fragmentation Syndrome

CHAPTER OUTLINE

- ❖ Introduction
- ❖ Classification
- ❖ Laboratory Findings

INTRODUCTION

Red blood cells, when subjected to physical or mechanical trauma in the circulation may undergo fragmentation. This causes intravascular hemolysis and results in hemolytic anemias. These are known as fragmentation syndrome.

CLASSIFICATION

Hemolytic anemia resulting from trauma to RBCs is classified according to the site of hemolysis as **macroangiopathic** (large vessels) or **microangiopathic** (capillaries) (Table 11.1). Mechanical fragmentation is either due to alteration of the endothelial surface of blood vessels or disturbances in blood flow causing turbulence and increased shear forces/stress.

Table 11.1: Hemolytic anemia resulting from trauma to RBCs

- **Macroangiopathic hemolytic anemia**
  - Prosthetic heart valve, defective heart valve, synthetic vascular graft
- **Microangiopathic hemolytic anemia (MAHA)**
  - Disseminated intravascular coagulation (DIC)
  - Thrombotic thrombocytopenic purpura (TTP)
  - Hemolytic-uremic syndrome (HUS)
  - Malignant hypertension
  - Systemic lupus erythematosus
  - Disseminated cancer
  - Hemolysis with elevated liver enzymes and low platelets-**HELLP**
- **Repetitive physical trauma**
  - March hemoglobinuria

- **Macroangiopathic hemolytic anemia** results most often due to direct red cell trauma from an abnormal vascular surface (e.g., prosthetic heart valve, synthetic vascular graft).
- **Microangiopathic hemolytic anemia (MAHA)** results from abnormal luminal narrowing in the microcirculation, most often due to the deposition of fibrin and platelets that cause turbulent blood flow.

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LABORATORY FINDINGS

They are similar in both macro and microangiopathic hemolytic anemias.

- **Hemoglobin:** Decreased. Anemia is mild to moderate.
- **Peripheral smear**:
  - **RBCs:** They show **fragmented red blood cells** (known as **schistocytes),** polychromasia, with or without nucleated red blood cells and microspherocytes. The fragmented red cells assume different shapes like "triangular cells", "burr cells," "helmet cells" (see Figs 42.3A and B) and are the hallmark of diagnosis of this group of hemolytic anemias.
  - **WBCs:** Count within normal limits or increased.
  - **Platelets:** Thrombocytopenia is often seen.

March Hemoglobinuria

This hemolytic disorder develops in athletes and soldiers following long distance running or marching, prolonged walking and vigorous exercise. Mechanical destruction of red cells occurs in the capillaries of the feet and results in transient hemoglobinemia and hemoglobinuria.

SUMMARY

- Hemolytic anemias due to mechanical trauma include macroangiopathic hemolytic anemias and MAHA.
- Macroangiopathic hemolytic anemias are due to trauma induced by prosthetic cardiac valves.
- MAHA results from red blood cells passing through the fibrin deposits in the microvasculature.
- Causes of MAHA include DIC, TTP, HUS, malignant hypertension and disseminated cancer.
- Both macro and microangiopathic hemolytic anemias are characterized by the presence of fragmented red blood cells known as schistocytes (like "triangular cells", "burr cells," "helmet cells") in the peripheral smear.

SELF-ASSESSMENT EXERCISES

I. Short Note

Microangiopathic hemolytic anemias

II. Multiple Choice Questions

1. **The fragmented RBCs are NOT seen in the peripheral smear of**
   A. Disseminated intravascular coagulation
   B. Malignant hypertension
   C. Hemolysis with elevated liver enzymes and low platelets
   D. Sickle cell anemia

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2. **The disorder associated with microangiopathic hemolytic anemia is**
   A. Henoch Schönlein purpura
   B. Chronic myelöid leukemia
   C. Thrombotic thrombocytopenic purpura (TTP)
   D. Immune thrombocytopenic purpura (TTP)
3. **The characteristic type of red cells seen in microangiopathic hemolytic anemia is**
   A. Spherocytes
   B. Microcytic hypochromic cells
   C. Target cells
   D. Triangular cells

ANSWERS

1. D **2.** C **3.** D

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Chapter 12
Paroxysmal Nocturnal Hemoglobinuria

Chapter Outline

- Definition
- Etiology and Pathogenesis
- Clinical Features
- Laboratory Findings

DEFINITION

Paroxysmal nocturnal hemoglobinuria (PNH) is an **acquired genetic defect** in the cell membrane due to **mutation in the hematopoietic stem cell.** Red cells in PNH are abnormally sensitive to complement-mediated intravascular hemolysis. Severity of hemolysis is proportional to the number of these abnormal red cells.

ETIOLOGY AND PATHOGENESIS

- PNH results from acquired **mutations in the phosphatidylinositol glycan-group A** (PIGA) gene in hematopoietic stem cells (Fig. 12.1). Since it is a hematopoietic stem cell disorder, PNH is not limited to red blood cells only, but also involves leukocytes and platelets. PIGA gene is X-linked, hence a single acquired mutation results in PNH.
- PIGA gene mutation results in **deficient synthesis of GPI-linked proteins** (in blood cells). These proteins anchor several proteins to the red cell membrane and some are responsible for complement degradation. These GPI-linked proteins are:
  - Decay–accelerating factor or CD55
  - Membrane inhibitor of reactive lysis or CD59, which is the most important and a potent inhibitor of C3. It prevents activation of the alternative complement pathway.
  - C8 binding protein.

  Main features: Two main features of PNH are:
  - **Intravascular hemolysis:** Deficiency of proteins that regulate complement renders the red cells susceptible to complement-mediated hemolysis. Chronic hemolysis without hemoglobinuria is more common than the paroxysmal and nocturnal hemolysis, despite the name (nocturnal). Nocturnal hemolysis is due to reduced pH of blood during sleep which enhances the activity of complement. Hemoglobin in acidic urine is converted into acid hematin which colors the **urine dark brown**. The loss of heme iron in the urine (hemosiderinuria) eventually causes iron deficiency.

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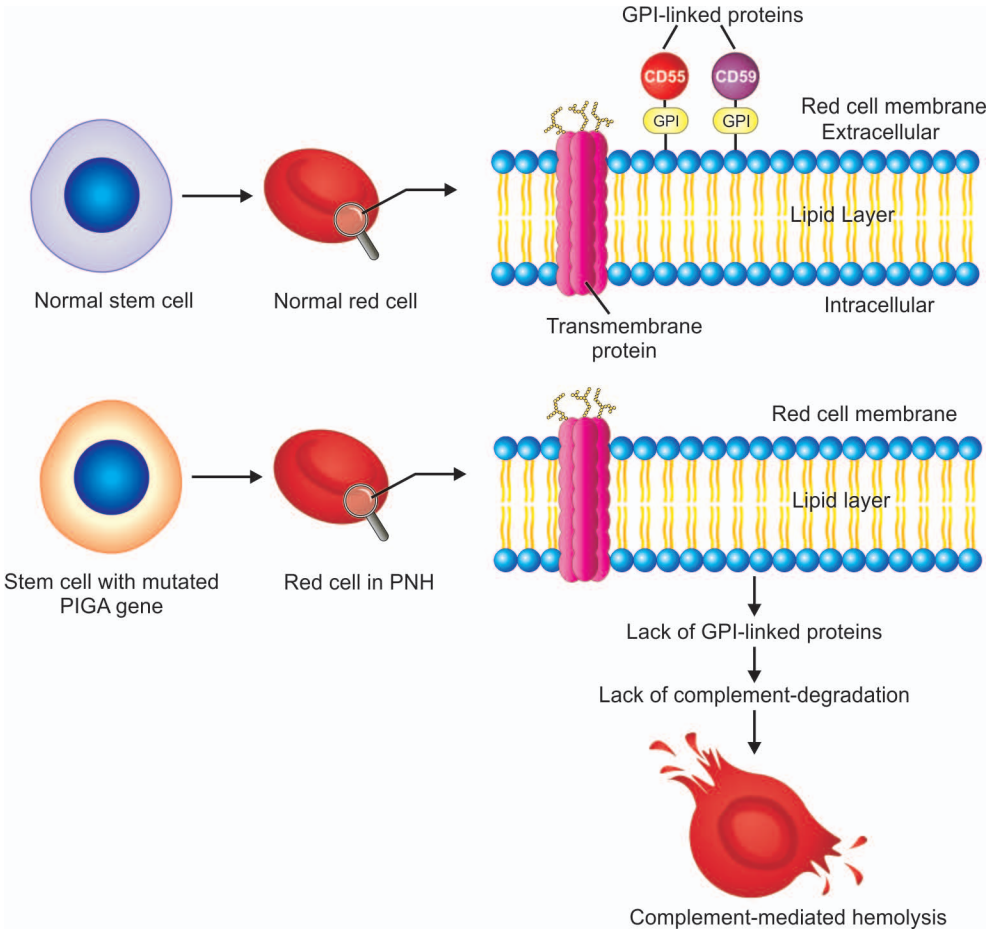


Fig. 12.1: Appearance of GPI-linked proteins on the normal red cell membrane. In PNH, red cells are deficient in GPI-linked proteins

- **Thrombosis:** Absence of GPI-linked proteins in platelets and absorption of NO by free hemoglobin predisposes to thrombosis. Thrombosis involving the hepatic, portal or cerebral veins is often the leading cause of death.

PNH may develop as a primary disorder or evolve from pre-existing aplastic anemia. This may be due to genetic damage of hematopoietic stem cells.

CLINICAL FEATURES

Since the disease is of variable severity, clinical course is variable lasting few years to 15 years. Death in most of the cases is because of thrombosis, especially of hepatic veins (Budd-Chiari Syndrome). PNH may be associated with myelodysplastic syndrome (stem cell disorder), which may subsequently progress to acute myelogenous leukemia and prognosis is poor in these patients. Bone marrow transplantation is curative.

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LABORATORY FINDINGS

Peripheral Blood

- **Hemoglobin:** Decreased.
- **Hematocrit:** Decreased.
- **Reticulocyte count:** Increased.
- **Peripheral smear**
  - **RBCs:** Show normocytic normochromic anemia with polychromatophilia (due to reticulocyte response). Severe and repeated episodes of hemoglobinuria over a period of years lead to microcytic hypochromic anemia due to iron deficiency.
  - **WBCs:** Total leukocyte count is either normal or reduced with mild neutropenia.
  - **Platelets:** Appear either normal or reduced in number.
- **NAP Score:** Neutrophil alkaline phosphatase score is reduced (normal 40 to 100), because NAP is a GPI linked protein.
- Features of **intravascular hemolysis** like hemoglobinemia, hemoglobinuria and hemosiderinuria are present.

Ham's Acidified Serum Test and Sucrose Hemolysis Test

In PNH, patient's red cells undergo lysis when incubated with acidified serum (Ham test) or sugar (sucrose hemolysis test). This is due to enhanced complement binding to red cells.

Flow Cytometry

Flow cytometry detects red cells that are deficient in GPI-linked proteins (CD55 and CD59) and is useful for diagnosis of PNH.

SUMMARY

- PNH is an acquired blood cell disorder due to mutations in the phosphatidylinositol glycan-group A (PIGA) gene in hematopoietic stem cells.
- There is deficiency of GPI linked proteins, which normally protect the cells against complement mediated lysis.
- Two characteristic features of PNH are intravascular hemolysis and thrombosis.
- Flow cytometric analysis of peripheral blood for CD55 and CD59 is the definitive test for diagnosis of PNH.

SELF-ASSESSMENT EXERCISES

I. Short Note

Paroxysmal nocturnal hemoglobinuria

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II. Multiple Choice Questions

1. **Paroxysmal nocturnal hemoglobinuria is**
   A. An acquired mutational disorder of stem cell
   B. A congenital red cell disorder
   C. An autoimmune disorder
   D. Due to deficient synthesis of β-globin chain
2. **The following hemolytic anemia is due to acquired intracorpuscular defect**
   A. Cold hemagglutinin disease
   B. Paroxysmal cold hemoglobinuria
   C. Paroxysmal nocturnal hemoglobinuria
   D. Autoimmune hemolytic anemia
3. **The hemolytic anemia cause by non-immune mechanism is**
   A. Cold hemagglutinin disease
   B. Transfusion of incompatible blood
   C. Paroxysmal cold hemoglobinuria
   D. Paroxysmal nocturnal hemoglobinuria
4. **Following features are those of paroxysmal nocturnal hemoglobinuria EXCEPT**
   A. Acquired clonal disorder of stem cells
   B. Positive Ham's test
   C. Risk of venous thrombosis
   D. Predominant extravascular hemolysis
5. **In paroxysmal nocturnal hemoglobinuria, the laboratory test which identifies sensitivity of RBC to complement is**
   A. Ham's test
   B. Hess test
   C. Heinz body test
   D. Direct Coombs test

ANSWERS

1. A **2.** C **3.** D **4.** D **5.** A

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CHAPTER 13 Approach to Hemolytic Anemias

Approach to the diagnosis of hemolytic anemia is a three step process.

- Diagnosis of anemia as hemolytic (Fig. 13.1)
- Determine the cause of the hemolysis (Fig. 13.2)
  - Acquired
  - Hereditary
- Confirmation of the disease by specific test

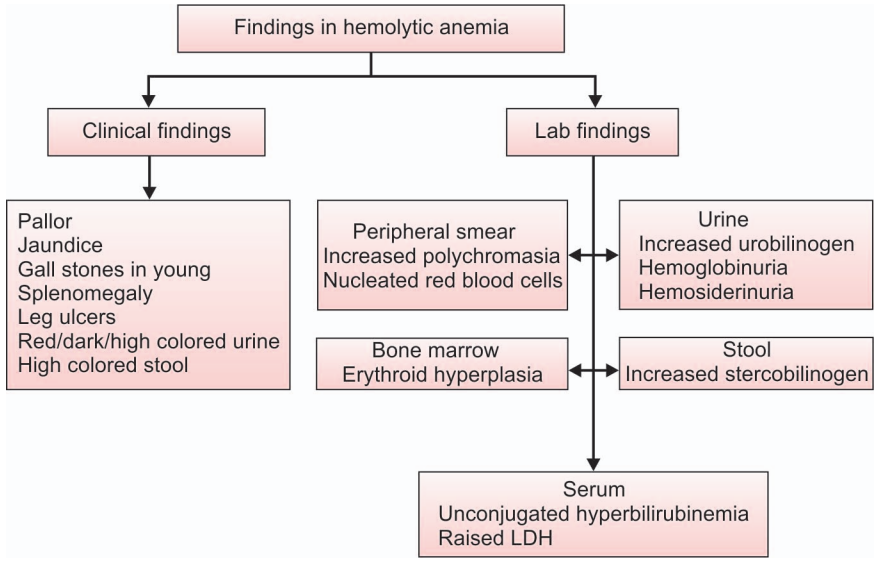


Fig. 13.1: Steps involved in the diagnosis of hemolytic anemia

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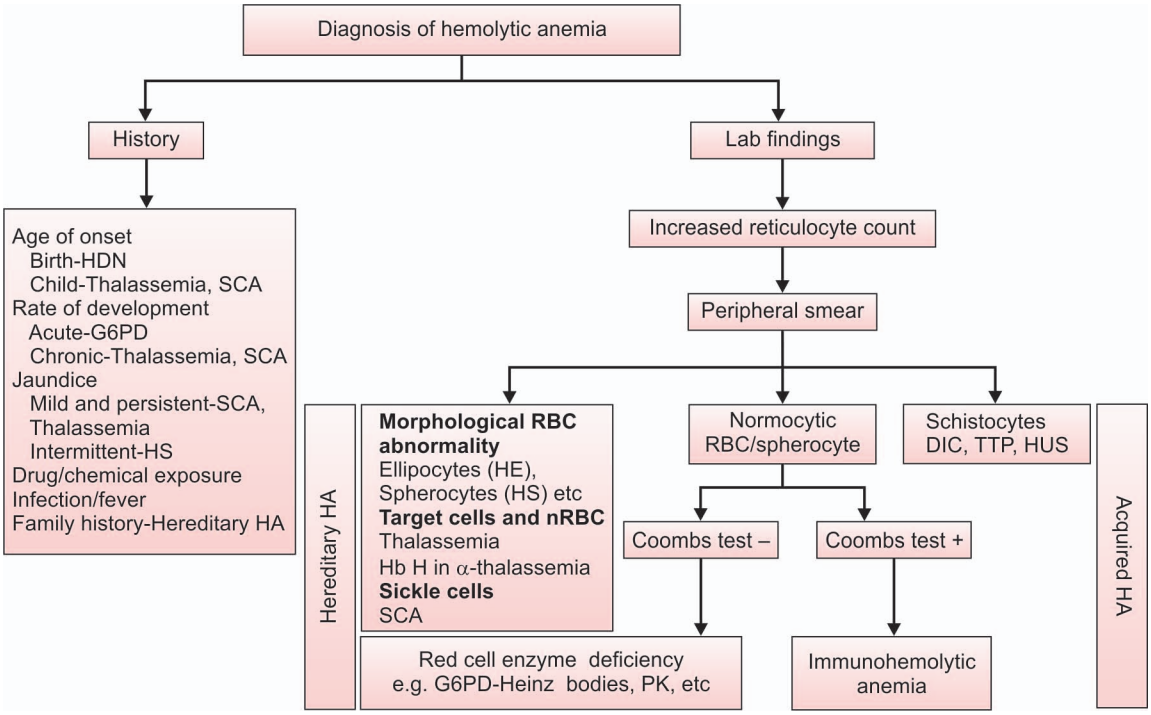


Fig. 13.2: Steps involved in identifying the causes of hemolytic anemias

SELF-ASSESSMENT EXERCISES

I. Essay

Discuss the approach to a patient with hemolytic anemia

II. Multiple Choice Questions

1. **Example of acquired hemolytic anemia is**
   - A. Sickle cell anemia
   - B. Beta thalassemia
   - C. Thrombotic thrombocytopenic purpura
   - D. Hemoglobin H Disease
2. **Coombs test is positive in**
   - A. Alpha thalassemia
   - B. Sickle cell anemia
   - C. Hereditary spherocytosis
   - D. Immunohemolytic anemia

ANSWERS

1. C **2.** D

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Chapter 14

Aplastic Anemia

Chapter Outline

- Definition
- Etiology
- Pathogenesis
- Clinical Features
- Laboratory Findings

DEFINITION

Aplastic anemia is a hematopoietic stem cell (HSC) disorder characterized by **pancytopenia** (anemia, neutropenia and thrombocytopenia) associated **with hypocellular bone marrow** (less than 30% cellularity). Hematopoietic stem cells in the bone marrow are replaced by fat cells and result in deficient production of all the blood cells.

ETIOLOGY

Aplastic anemia results from injury to hematopoietic stem cells in the bone marrow. The most common causes associated with aplastic anemia are shown in Table 14.1.

Acquired

- **Chemical Agents:** Certain drugs and agents (cancer chemotherapy drugs and the organic solvent benzene) cause marrow damage, which is dose related and reversible, when the offending agent is withdrawn.
- **Idiosyncratic:** Some chemicals and drugs cause marrow suppression due to an idiosyncratic reaction to the drugs (e.g. chloramphenicol, phenylbutazone).
- **Physical Agents:** Whole-body irradiation can destroy hematopoietic stem cells. The damage is dose-dependent and follows either therapeutic irradiation or nuclear accidents.
- **Viral Infections:** Viral hepatitis of unknown types; CMV, HIV and EBV is associated in few cases with aplastic anemia.
- **Idiopathic:** Most cases (approximately 65%) are idiopathic without any identifiable specific initiating etiological factor.

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Table 14.1: Common causes of aplastic anemia

ACQUIRED

Idiopathic

- Acquired defects in stem cell
- Immune mediated

Secondary

Chemical Agents

- Cytotoxic drugs
  - Alkylating agents
  - Antimetabolites
- Benzene
- Inorganic arsenicals
- Chloramphenicol

Idiosyncratic

- Chloramphenicol
- Phenylbutazone
- Penicillamine
- Carbamazepine
- Gold salts
- Organic arsenicals
- Methyl-phenyl-ethyl hydantoin

Physical Agents

- Whole-body irradiation

Viral Infections

- Hepatitis (unknown type)
- Epstein-Barr virus infections
- Cytomegalovirus infections
- Herpes zoster (Varicella zoster)
- HIV

INHERITED

- Fanconi anemia
- Telomerase defects

6"I"s of aplastic anemia

Idiopathic
Ingestion of drugs and chemicals
Idiosyncratic
Irradiation
Infections
Inherited

Inherited

- *Fanconi anemia* is a rare autosomal recessive inherited disorder.
- *Telomerase defects:* Telomerase is required for cellular immortality and limitless replication of cells. Hence, defects in telomerase activity can cause premature hematopoietic stem cell exhaustion and marrow aplasia.

PATHOGENESIS

The exact pathogenesis of aplastic anemia is not known. The mechanism of aplastic anemia falls under three main categories: direct damage, immune-mediated destruction of marrow progenitor cells and primary stem cell abnormality (Fig. 14.1).

- **Direct damage to the hematopoietic stem cells and progenitor cells:** This may alter the ability of the cell to proliferate or differentiate. The damage may be the result of known agent such as cytotoxic drugs, irradiation and viruses or unknown agent.

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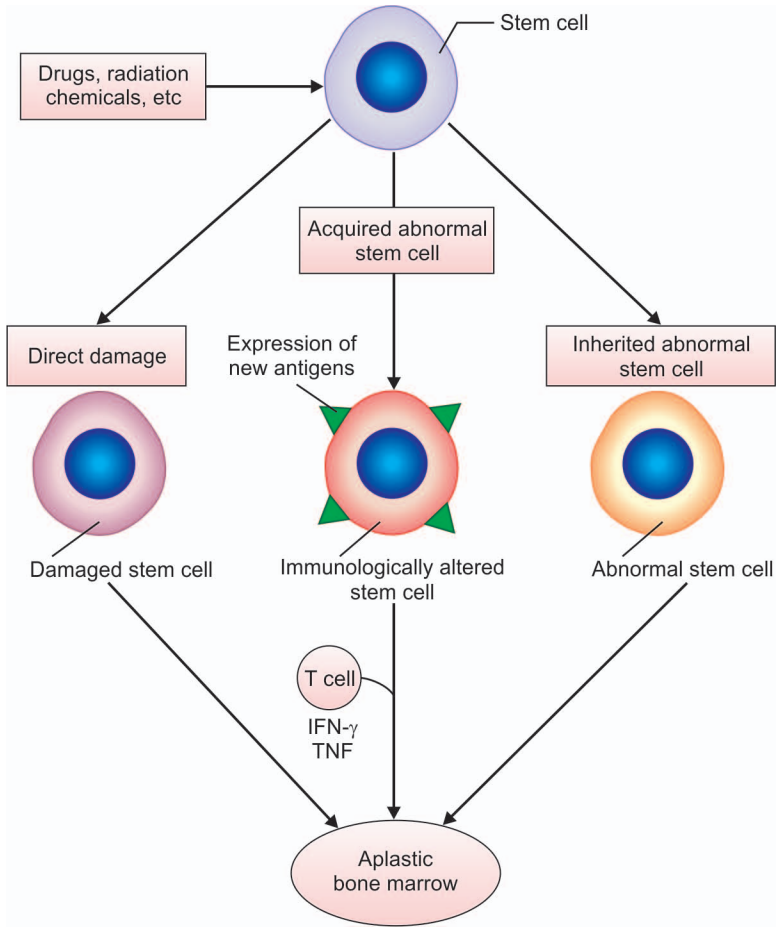


Fig. 14.1: Pathogenesis of aplastic anemia

- **Immune-mediated destruction:** Acquired aplastic anemia is considered as an immune-mediated syndrome in which destruction of stem and progenitor cells occurs in the following ways:
  - Chemical, viral or unidentified environmental antigens may produce acquired stem cell abnormality. Abnormal stem cells might express neoantigens and alter the stem cell antigenicity.
  - Immunologically altered stem cell provokes a cellular immune response and activation of cytotoxic T lymphocyte.
  - Activated T_H1 cells produce cytokines such as interferon-γ (IFNγ) and TNF.
  - The cytokines suppress and destroy hematopoietic progenitors.
- **Primary stem cell abnormality:** Aplastic anemia may also be due to inherited defect in the stem cells.

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CLINICAL FEATURES

- Aplastic anemia can occur at any age of both sexes.
- Insidious in onset and the initial presenting feature depends on the hematopoietic cell line predominantly affected.
  - Anemia: This causes progressive weakness, pallor and dyspnea.
  - Neutropenia: Presents as frequent (mucocutaneous bacterial infections) or fatal infections.
  - Thrombocytopenia: Results in bleeding manifestations in the form of petechiae, bruises and ecchymoses.

LABORATORY FINDINGS

Peripheral Blood

Blood and bone marrow picture is similar in idiopathic and secondary aplastic anemia.

- **Hemoglobin:** Decreased.
- **PCV:** Decreased.
- **Reticulocyte count:** Reticulocyte count is markedly decreased (varies from 0.5 to 1%). It is known as reticulocytopenia and is a characteristic feature.
- **Peripheral smear:** Shows pancytopenia, i.e. decreased red cells, neutrophils and platelets. In early stages, single cell line shows decreased count or bicytopenia.
  - **RBCs:** They are typically normocytic and normochromic, although slight macrocytosis may be occasionally present.
  - **WBCs:** Total leukocyte count is decreased. Neutrophils are markedly diminished and neutropenia is a reflection of the severity of aplasia. In the initial stages, lymphocytes are normal in number but with progression of the disease their count also decreases.
  - **Platelets:** Count is decreased.

Bone Marrow

Marrow aplasia is best appreciated in a bone marrow (trephine) biopsy as the marrow involvement may be focal.

- **Cellularity:** Marked hypocellularity with replacement of more than 70% of the marrow cells by fat.
- **Hematopoiesis:** Paucity of all erythroid, myeloid and megakaryocytic precursors. Initial stages may show focal areas of hematopoiesis.
- **Other cells:** Lymphocytes and plasma cells are prominent.

***Splenomegaly* is absent** and in its presence the diagnosis of aplastic anemia should not be made.

Diagnosis: Diagnosis is made with **peripheral blood** and **bone marrow biopsy findings.**

Differential diagnosis: Aplastic anemia should be distinguished from other causes of pancytopenia (Table 14.2) such as "aleukemic" leukemia and myelodysplastic syndromes,

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Table 14.2: Causes of pancytopenia

- Decreased bone marrow function
  - Aplastic anemia
    - Idiopathic
    - Secondary
    - Inherited
  - Myelodysplastic syndromes
  - Bone marrow infiltration with
    - Leukemia
    - Lymphoma
    - Myeloma
    - Tumors (carcinoma)
    - Granulomatous diseases (e.g. tuberculosis, sarcoidosis)
  - Nutritional deficiencies:
    - Megaloblastic anemia (vitamin B_{12} and folic acid deficiency)
  - Paroxysmal nocturnal hemoglobinuria
  - Myelofibrosis (rare)
  - Hemophagocytic syndrome
- Increased peripheral destruction
  - Hypersplenism

which have similar clinical manifestations. In aplastic anemia, the marrow is usually markedly hypocellular, with prominence of lymphocytes and plasma cells and in contrast the myeloid neoplasms have abnormal myeloid progenitors.

Prognosis: Prognosis of aplastic aplasia is unpredictable. Withdrawal of toxic drugs can lead to recovery in some cases. Spontaneous remission in idiopathic cases is unfortunately uncommon. Bone marrow transplantation is the treatment of choice. Immunosuppressive therapies will be helpful in older patients.

Fanconi Anemia: It is a rare inherited cause of aplastic anemia. Peripheral blood in aplastic anemia shows pancytopenia with reticulocytopenia. Bone marrow is markedly hypocellular with paucity of erythroid, myeloid and megakaryocytic precursors. Marrow hypofunction is often associated with multiple congenital anomalies (hypoplasia of the kidney and spleen and bone anomalies).

SUMMARY

- Aplastic anemia is characterized by reduction or cessation of production of blood cells.
- Aplastic anemia may be acquired or congenital. The acquired aplastic anemia may be idiopathic or secondary to drugs, chemicals, radiation, infections, etc.
- In acquired aplastic anemia the bone marrow aplasia is due to destruction of hematopoietic stem cells by direct or indirect damage. The indirect damage may result in expression of neoantigens and their subsequent destruction by immune mechanism.
- The bone marrow aplasia is best appreciated in a bone marrow (trephine) biopsy.
- Fanconi anemia is a rare inherited cause of aplastic anemia.

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SELF-ASSESSMENT EXERCISES

I. Short Note

Aplastic anemia

II. Multiple Choice Questions

1. **Aplastic anemia is NOT caused by**
   A. Benzene
   B. Whole body irradiation
   C. Chloramphenicol
   D. Bacterial endocarditis
2. **The following is NOT true with regard to acquired aplastic anemia**
   A. Preceded by viral hepatitis
   B. Absence of splenomegaly
   C. Preceded by paroxysmal nocturnal hemoglobinuria
   D. Increased reticulocyte count

ANSWERS

1. D **2.** D

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Chapter 15 Pure Red Cell Aplasia

Chapter Outline

- Definition
- Etiology
- Clinical Features
- Laboratory Findings

DEFINITION

Pure red cell aplasia (PRCA) is a disorder in which there is **primary bone marrow failure** due to **selective suppression of erythroid precursors** in the bone marrow. This results in anemia with reticulocytopenia. The myeloid and megakaryocytic lineages are unaffected.

ETIOLOGY

Majority of the PRCA are acquired and very few cases are of the congenital type.

- ***Congenital:*** Diamond-Blackfan syndrome is a congenital type of PRCA that appears in the first year of life. It is associated with defective erythroid precursors.
- ***Acquired:*** PRCA may occur in association with
  - Neoplasms
    - Thymoma
    - Large granular lymphocytic leukemia
  - Exposure to drugs
  - Autoimmune disorders
  - Parvovirus infection

  Except for parvovirus infection, most of the PRCA are of autoimmune origin.

CLINICAL FEATURES

- Acquired PRCA may present either as an **acute** self-limited illness or a **chronic** relapsing disease.
- Acute PRCA is often due to parvovirus B19, which selectively infects and destroys red cell progenitors. This may be clinically asymptomatic except in patients with hemolytic anemia (e.g. hereditary spherocytosis, sickle cell anemia). Such patients may present with sudden worsening of anemia due to aplastic crisis.
- In the immunocompromised (HIV infection), infection may persist, leading to chronic red cell aplasia.

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LABORATORY FINDINGS

Peripheral Blood

- **Hemoglobin:** Decreased. Patients develop moderate to severe degree of anemia.
- **Peripheral smear:** Shows macrocytosis. Despite increased EPO, there is no accompanying reticulocytosis.

Bone Marrow

- **Cellularity:** Normal cellularity, but with a selective absence of erythroid precursors.
- **M:E ratio:** May vary from 19:1 to 49:1
- **Erythropoiesis:** In PRCA secondary to parvovirus B19, intranuclear viral inclusions in pronormoblasts can be seen.
- **Myelopoiesis:** Normal
- **Megakaryopoiesis:** Normal

SUMMARY

- Pure red cell aplasia is a disorder of primary bone marrow failure due to suppression of only the erythroid precursors. It may be congenital (Diamond Blackfan anemia) or acquired.
- Acquired PRCA are seen in association with thymoma, following drugs, parvovirus infection or autoimmune.

SELF-ASSESSMENT EXERCISE

Multiple Choice Question

1. **Pure red cell aplasia may be associated with all of the following EXCEPT**
   A. Thymoma
   B. Autoimmune disorders
   C. Acute myeloid leukemia
   D. Parvovirus infection

ANSWER

1. C

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CHAPTER 17 Sideroblastic Anemia

CHAPTER OUTLINE

- ❖ Introduction
- ❖ Hereditary Sideroblastic Anemias
- ❖ Acquired Idiopathic Sideroblastic Anemia
- ❖ Congenital Dyserythropoietic Anemias

INTRODUCTION

Definition

Sideroblastic anemias are a rare heterogeneous group of refractory anemias characterized by:

- Presence of ring sideroblasts in the bone marrow aspirate.
- A dimorphic peripheral blood picture. Microcytic hypochromic red cells in hereditary form and macrocytic in the acquired forms of the disease mixed with normochromic cells.
- Tiny iron-containing inclusions called as Pappenheimer bodies in the red blood cells (stain positively by Prussian blue staining).
- Increased serum iron concentration and markedly increased storage iron.
- Ineffective erythropoiesis due to non-viable sideroblasts.

Classification

Sideroblastic anemias are classified mainly into:

- Hereditary sideroblastic anemia.
- Acquired sideroblastic anemia.

Types of Sideroblasts

Sideroblasts are the normoblasts in the marrow containing iron-positive (siderotic) granules demonstrated by Prussian blue staining. The various types of sideroblasts are shown in Table 17.1.

Table 17.1: Types of sideroblasts

Type	*Condition*	*Size of iron granules in cytoplasm*
Sideroblast	Normal	1-2 pinpoint sized, randomly distributed
Abnormal sideroblasts	Hemolytic anemias	1-5 pinhead sized, randomly distributed
Ring sideroblasts	Pathognomonic of sideroblastic anemias	Pinhead sized iron granules arranged around one-third or more of the nucleus or 5 or more granules forming a partial or complete perinuclear ring

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HEREDITARY SIDEROBLASTIC ANEMIAS

Hereditary sideroblastic anemias comprise a clinically, genetically and hematologically heterogeneous group of rare disorders. It may be inherited as X-linked or an autosomal (dominant or recessive) disorder. These patients generally have low levels of δ-aminolevulinic acid synthase (ALAS) enzyme in the normoblasts leading to defective synthesis of hemoglobin.

Clinical Features

- **Sex:** It predominantly affects males.
- ***Anemia:*** It may not manifest until adolescence.
- ***Iron overload*** *may result in:*
  - *Hepatomegaly*
  - *Splenomegaly*
  - *Impaired growth and development*
  - *Cardiac arrhythmias*

Laboratory Findings

Peripheral Blood

- **Red cell indices:**
  - **MCV:** Decreased
  - **MCH:** Decreased
- **Peripheral smear**
  - **RBCs:** Red cells show dimorphic blood picture with moderate degree of *anisopoikilocytosis.* There are *microcytic hypochromic red cells mixed with normocytic normochromic red cells*. Few red cells may show *basophilic stippling.* Occasional nucleated RBC may be found.
  - **WBCs and platelets:** Usually normal. However, leucopenia and thrombocytopenia may develop due to hypersplenism.
- **Reticulocyte count:** 0.2-1%.

Bone Marrow

- **Cellularity:** Hypercellular
- **Erythropoiesis:** Erythroid hyperplasia but is ineffective. The reaction may be normoblastic or micronormoblastic.
- **Iron:** Moderate to marked increase in bone marrow iron. *Ring sideroblasts* with partial/complete perinuclear ring of iron granules (Fig. 17.1) are characteristically present.

Other Findings

- Serum ferritin, serum iron and transferrin saturation are increased.

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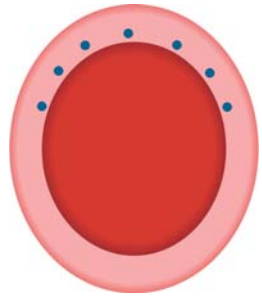


Fig. 17.1: Ring sideroblasts with partial perinuclear ring of iron granules

ACQUIRED IDIOPATHIC SIDEROBLASTIC ANEMIA

- The term primary or idiopathic is applied when other causes of sideroblastic anemia cannot be identified.
- Acquired idiopathic sideroblastic anemia (AISA) constitutes a subgroup of the myelodysplastic syndromes (Chapter 24), refractory anemia with ring sideroblasts (RARS). Majority present after 40 years of age.
- The dimorphic anemia has both hypochromic-microcytic and macrocytic red blood cells, and the MCV is usually high. At least 15% or more of erythroblasts (early and late forms) in bone marrow are ring sideroblasts.

Secondary (drug- or toxin-induced) Sideroblastic Anemia

This form of sideroblastic anemia is secondary to various agents that interfere with heme synthesis.

- ***Antituberculous drugs:*** Long-term therapy with isoniazid, cycloserine and pyrazinamide.
- ***Lead poisoning***: Lead interferes with heme synthesis by blocking the enzymes ALA synthase, ALA dehydratase and heme synthetase.
- ***Chloramphenicol:*** Sideroblastic anemia results probably due to inhibition of mitochondrial protein synthesis.
- ***Copper deficiency or zinc overload:*** This results in sideroblastic anemia and neutropenia. Copper chelators in large doses, such as penicillamine, can produce sideroblastic anemia.
- ***Ethanol-induced anemia:*** This is perhaps the most common of the reversible sideroblastic anemias. Folate deficiency, hypomagnesemia and hypokalemia are concomitant findings.
- ***Primary pyridoxine deficiency:*** It is often associated with malnutrition, is occasionally associated with sideroblastic anemia.

CONGENITAL DYSERYTHROPOIETIC ANEMIAS (CDA)

Congenital dyserythropoietic anemias (CDAs) are characterized by ineffective erythropoiesis and marrow erythroid multinuclearity. Three common types are recognized.

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CDA-I is characterized by macrocytic anemia. Bone marrow shows megaloblastic erythropoiesis with internuclear chromatin bridges.

CDA-II is the most common form and presents with normocytic normochromic anemia. Bone marrow shows normoblastic hyperplasia with binuclear and multinuclear normoblasts. CDA-II is distinguished from the others because it has a positive acidified serum test and a negative sucrose hemolysis test. It is also known as hereditary erythroblastic multinuclearity with positive acidified serum test (HEM-PAS).

CDA-III has giant erythroid precursors, with more pronounced multinuclearity (gigantoblasts) and a macrocytic anemia. In contrast to CDA-I and CDA-II, which are autosomal recessive, CDA-III has autosomal dominant inheritance.

SUMMARY

- Sideroblastic anemias are a rare heterogeneous group of refractory anemias characterized by presence of ring sideroblasts in the bone marrow aspirate.
- Sideroblastic anemias are classified mainly into hereditary and acquired types.
- Ring sideroblasts are pathognomonic of sideroblastic anemias.
- Patients with hereditary sideroblastic anemias generally have low levels of δ-aminolevulinic acid synthase (ALAS) enzyme in the normoblasts leading to defective synthesis of hemoglobin.
- Acquired idiopathic sideroblastic anemia constitutes a subgroup of the myelodysplastic syndromes referred to as refractory anemia with ring sideroblasts (RARS).

SELF-ASSESSMENT EXERCISE

Multiple Choice Questions

1. **The feature NOT characteristic of sideroblastic anemia is**
   A. Presence of ring sideroblasts in the bone marrow.
   B. Microcytic hypochromic red cells in hereditary form
   C. Pappenheimer bodies in the red blood cells
   D. Decreased storage iron.
2. **The drug which does NOT cause sideroblastic anemia is**
   A. Chloramphenicol
   B. δ-aminolevulinic acid synthase
   C. INH
   D. Penicillamine

ANSWERS

1. D **2.** B

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CHAPTER 18 Polycythemia

CHAPTER OUTLINE

- ❖ Introduction
- ❖ Polycythemia Vera

INTRODUCTION

Definition

Polycythemia, or erythrocytosis, refers to an increase in the RBC mass, usually with a corresponding increase in hemoglobin level. The increase in red cells can be absolute or relative.

Classification

A pathophysiologic classification of polycythemia is given in Table 18.1.

Table 18.1: Pathophysiologic classification of polycythemia

Absolute

Primary (low erythropoietin level)
- Polycythemia vera

Secondary (high erythropoietin level)
- Physiologically appropriate
  - Compensatory
  - Lung disease
  - Living in high-altitude
  - Cyanotic heart disease (Tetralogy of Fallot)
- Physiologically inappropriate (with increased erythropoietin)
  - Paraneoplastic: Erythropoietin-secreting tumors (e.g. renal cell carcinoma, uterine leiomyoma, hepatocellular carcinoma)

Relative

Reduced plasma volume
- Hemoconcentration (dehydration due to diarrhea, vomiting)
- Gaisböck syndrome (spurious polycythemia)

- **Relative polycythemia** is characterized by decreased plasma volume with a normal red cell mass.
  - They may result from dehydration following prolonged vomiting, diarrhea or excessive use of diuretics.
  - Gaisböck syndrome (spurious polycythemia) is seen in middle-aged, overweight, hypertensive and anxious ("stressed") individuals, and hence called as stress polycythemia. The cause is not known.

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- **Absolute polycythemia** is characterized by a true increase in total red cell mass and can be sub classified as primary and secondary.
  - **Primary polycythemia** or **polycythemia vera (PV):** It results from an intrinsic abnormality of the hematopoietic stem cells. PV is considered as one of the myeloproliferative neoplasm (Table 25.1).
  - **Secondary polycythemia:** It results as a compensatory response of red cell progenitors to an increase in EPO secretion. The erythropoietin secretion may be physiological as a response to general chronic tissue hypoxia or it may be pathological like in paraneoplastic syndromes.

POLYCYTHEMIA VERA

Definition

Polycythemia vera (PV) is an acquired myeloproliferative neoplasm arising from malignant transformation of hematopoietic stem cell. It is associated with mutations of erythropoietin receptor gene that lead to autonomous erythropoietin-independent proliferation of red cell progenitors.

- It is characterized by increased, uncontrolled marrow production of red cells, granulocytes and platelets (panmyelosis). This leads to erythrocytosis (polycythemia) and or granulocytosis and thrombocytosis in the peripheral blood. But erythrocytosis is responsible for most of the clinical symptoms.

Molecular Pathogenesis

- PV derives from malignant transformation of progenitor hematopoietic stem cell.
- **Normal signaling through erythropoietin receptor (Fig. 18.1):** Normal progenitor hematopoietic stem cells have an erythropoietin receptor (EPOR) lacking intrinsic tyrosine kinase activity. Binding of erythropoietin with its receptors (EPOR) results in interaction of a protein kinase called JAK with the cytoplasmic side of the EPOR receptor. The receptor gets phosphorylated and initiates activators of transcription protein STAT (signal transducers and activation of transcription). STAT directly binds to DNA and results in proliferation and differentiation of erythroid progenitors. This process is self-regulated by activated transduction molecule HCP (hematopoietic cell phosphatase). HCP binds to C-terminal of the erythropoietin receptor, which has negative regulatory function and turns off the signaling.
- **Signaling of erythropoietin receptor in PV:** The erythroid progenitor cells in PV have mutated EPOR gene and lack the C-terminal of the erythropoietin receptor. This causes activation of erythropoietin receptor even without erythropoietin, due to constitutive JAK signaling. The STAT produced, in turn causes continuous proliferation and is responsible for the increased red cell mass.
- Thus, there is an erythropoietin-independent growth of red cell progenitors with low serum erythropoietin levels in PV. This is in contrast to high erythropoietin levels in secondary forms of absolute polycythemia.

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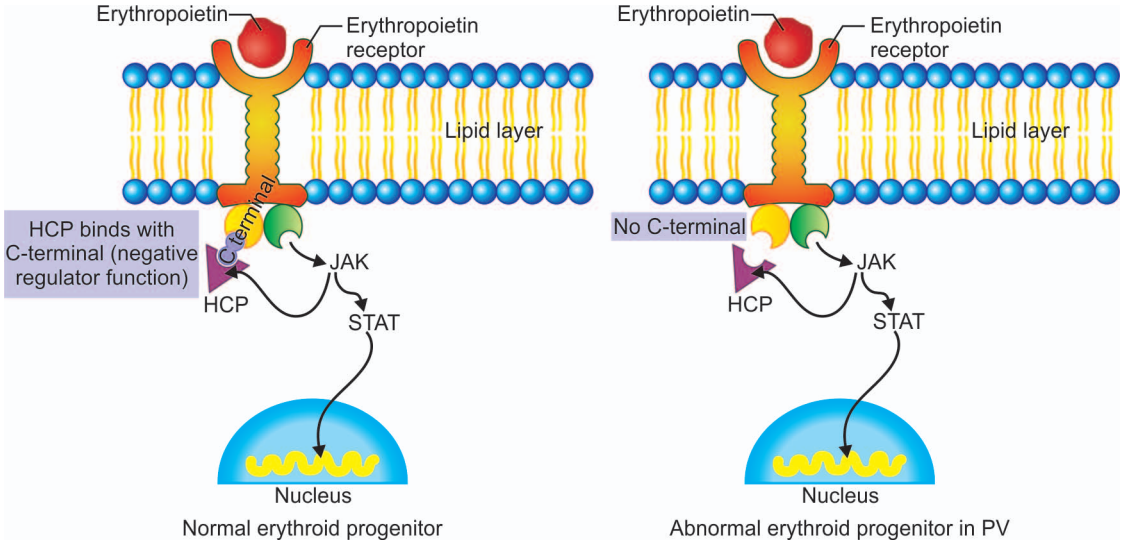


Fig. 18.1: Molecular pathogenesis of polycythemia vera

- In PV the proliferative activity of progenitor cells is less than in CML (another myeloproliferative neoplasm). This may be due to quantitatively weaker or qualitatively different JAK signals in contrast to stronger signals produced by BCR-ABL in CML.

Clinical Features

- Usually appears insidiously, in late middle age (median age at onset is 60 years). Most symptoms are due to the increased red cell mass and hematocrit.
- Plethora (a morbid condition due to excess of RBCs in the blood or increase in the quantity of blood) and cyanosis due to stagnation and deoxygenation of blood in peripheral vessels are early findings. Headache, dizziness and visual problems result from vascular disturbances in the brain and retina.
- The elevated hematocrit results in increased blood viscosity and sludging. These factors, along with thrombocytosis and abnormal platelet function, predispose to thrombotic episodes. These episodes may manifest as deep venous thrombosis, myocardial infarction, thrombosis of hepatic veins (producing Budd-Chiari syndrome), the portal and mesenteric vein thrombosis (leading to bowel infarction), and the venous sinuses of the brain (leading to hemorrhagic strokes).

  Phases: The clinical course tends to proceed as a series of phases.
  - **Proliferative phase:** There is erythroid proliferation with increased red cell mass.
  - **Spent phase:** In 10% of cases, excessive proliferation of erythroid cells ceases, resulting in stable or decreased erythrocyte mass.
  - **Myelofibrosis:** About 10% of cases progress to myelofibrosis (postpolycythemic myelofibrosis with myeloid metaplasia). Extramedullary hematopoiesis in spleen leads to splenomegaly.
  - **Acute myelogenous leukemia:** May develop in 2 to 5% of cases.

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Laboratory Findings

Peripheral Blood

- **Hemoglobin:** Increased. It is more than 18.5 gm/dL in men and 16.5 gm/dL in women.
- **Hematocrit:** Increased to about 60%.
- **Red cell count : Increased and usually about 6 million/cu mm (6×10^{12}/L)**
- **White cell count:** Normal or increased
- **Platelet count:** Normal or increased
- **Peripheral smear:**
  - **RBCs:** Show normocytic normochromic picture.
  - **WBCs:**
    - Total leukocyte count is increased.
    - There is mild to moderate leukocytosis.
    - Neutrophils are morphologically normal.
    - Basophils often increased
    - NAP (LAP) score is increased to 150-300 (Normal 40-100)
  - **Platelets:** The platelets are often abnormally large and functionally defective.

Bone Marrow

- **Cellularity:** Hypercellular.
  Hyperplasia of all elements (trilineage hyperplasia/panmyelosis) namely erythroid, myeloid and megakaryocytic series with prominence of erythroid precursors in the bone marrow.

Bone Marrow Biopsy

It shows increased reticulin fibers and fibrosis.

- Moderate to marked increase in marrow reticulin fibers is observed in approximately 10% of the patients.
- Late in the course, it can progress to a spent phase characterized by extensive marrow fibrosis that displaces hematopoietic cells.
- This is associated with extramedullary hematopoiesis in the liver and spleen that causes **hepatosplenomegaly**.
- Uncommonly, transformation to AML also occurs.

Other Findings

- **Arterial oxygen saturation (pO2):** It is normal (75-100 mm Hg) and is useful for **differentiating it from secondary** polycythemia.
- **Erythropoietin levels: Decreased** in contrast to secondary polycythemia.
- **Serum vitamin B_{12} and uric acid:** They are increased indicating increased cell turnover.
- **JAK2 V617F or other functionally similar mutation:** They can be demonstrated.

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SUMMARY

- Polycythemia or erythrocytosis, refers to an increase in the RBC mass. Polycythemia may be absolute or relative.
- Polycythemia vera is one of the primary causes of absolute polycythemia.
- PV is characterized by increased red cell, leukocyte and platelet count. The spleen and liver are enlarged because of extramedullary hematopoiesis.
- Symptoms of PV are mainly due to increased hematocrit. Elevated hematocrit, thrombocytosis and abnormal platelet function predispose to both thrombosis.

SELF-ASSESSMENT EXERCISES

I. Short Note

Polycythemia

II. Multiple Choice Questions

1. **Molecular pathogenetic feature characteristic of polycythemia vera is**
   A. RB mutation
   B. MYC mutation
   C. RAS mutation
   D. JAK2 mutation
2. **The following does NOT lead to secondary polycythemia**
   A. Nephrotic syndrome
   B. Renal cell carcinoma
   C. Tetralogy of Fallot
   D. Chronic obstructive pulmonary disease
3. **The hematological disorder that does NOT cause hemosiderosis is**
   A. Beta-thalassemia
   B. Polycythemia vera
   C. Anemia of chronic disease
   D. Autoimmune hemolytic anemia
4. **Polycythemia vera is distinguished from secondary polycythemia by**
   A. Estimation of hematocrit
   B. RBC count
   C. Measuring arterial oxygen saturation
   D. Hemoglobin estimation

ANSWERS

1. D **2.** A **3.** B **4.** C

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SECTION 2

Disorders of White Cells

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Chapter 19
Quantitative and Qualitative Disorders of White Blood Cells

Chapter Outline

- Introduction
- Classification of WBC Disorders
- Quantitative Disorders of Leukocytes
- Qualitative Disorders of Leukocytes

INTRODUCTION

White blood cells (WBCs) or leukocytes include myeloid cells and lymphoid cells.

- Myeloid cells consist of neutrophils, eosinophils, basophils and monocytes.
- Lymphoid cells include small and large lymphocytes and natural killer (NK) cells.

WBCs mediate host defense against infection and modulate the immune response. Myeloid cells along with erythrocytes and platelets are derived from a common myeloid stem cell precursor (CMP).

Types of Normal White Blood Cells (Fig. 19.1)

Neutrophils

Neutrophils constitute 40 to 70% of circulating WBCs. Neutrophils have segmented nuclei and show 2 - 5 lobes. Their fine purple cytoplasmic granules contain various enzymes. Neutrophils stain positive for myeloperoxidase (MPO), leukocyte alkaline phosphatase (LAP) and chloroacetate esterase. They constitute first line of defense against infections.

- **Hypersegmented neutrophils (macropoly**s): Neutrophils having six or more than 6 nuclear lobes are termed hypersegmented neutrophils. They are found in macrocytic anemia and myelodysplastic syndromes (MDS).

Eosinophils

Eosinophils constitute 1 to 6% of circulating WBCs. They have a bilobed (spectacle shaped) nucleus and the cytoplasm shows specific orange/red colored coarse granules. These granules are composed of major basic protein (MBP), peroxidase, other lysosomal enzymes and Charcot-Leyden crystal protein. Immunoglobulin E receptors present on their surface play a role in allergic reactions and killing parasites.

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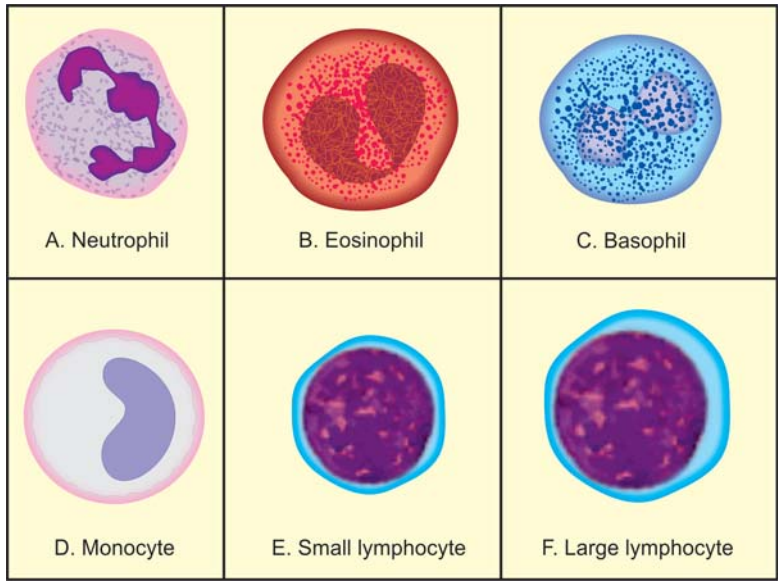


Fig. 19.1: Types of white blood cells

Basophils

Basophils constitute 0.3 to 1% of circulating WBCs. They have a multilobed nucleus which is almost covered by dark staining basophilic granules. When basophils are activated they release histamine and other cytokines from their granules, which stain positive for peroxidase. Basophils play a major role in type I hypersensitivity reaction and also defense against parasites.

Monocytes

Monocytes comprise 2 to 10% of circulating WBCs. They have abundant pale grayish blue cytoplasm. The nucleus is reniform or lobated, round to oval. In inflammation, they migrate to tissues and differentiate into macrophages.

- Main functions of macrophages are:
  - Phagocytosis of pathogens and noxious agents.
  - Demolition of debris and to facilitate wound healing.
  - Removal of senescent RBCs in the reticuloendothelial system (spleen, liver and bone marrow).
  - Presentation of antigen to T lymphocytes.
  - Storage function.

Lymphocytes

Lymphocytes constitute 20 to 40% of the circulating white cells. The lymphocytes are subdivided into **small** and **large** depending on their size and **B**, **T** lymphocytes and **NK cells** depending on their origin. In the peripheral blood, majority of them are small lymphocytes with few large lymphocytes. In tissue sections, it is usually not possible to distinguish T from B

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lymphocytes. But T cells show an irregularly contoured nuclear membrane and pale, clear cytoplasm.

- **Small lymphocytes**: Their size is approximately that of mature red cell (7 to 8 µm) and is used as comparator for assessing the size of RBCs as normocytic, microcytic or macrocytic. They have a thin rim of pale blue cytoplasm and nuclear chromatin is coarse.
- **Large lymphocytes:** These are 14 to16 µm in size and have central or eccentrically placed nucleus with condensed nuclear chromatin and clear pale blue cytoplasm.
- **Large granular lymphocyte:** These are natural killer (NK) cells or cytotoxic T cells. They are large and have abundant cytoplasm containing a few azurophilic granules (see Fig. 29.3).

Functions of lymphocytes: They are involved in immune regulation.

Normal Differential Leukocyte Count (DLC)

DLC is one of the routine, useful and important investigations. The normal range of DLC in an adult is presented in Table 19.1.

Table 19.1: Normal range of different leukocytes in an adult

Type of white blood cell	*Normal range*
Neutrophils	40-70% (2.0-7.0 × 10^9/L)
Lymphocytes	20-40% (1.0-3.0 × 10^9/L)
Monocytes	2-10% (0.2-1.0 × 10^9/L)
Eosinophils	1-6% (0.02-0.5 × 10^9/L)
Basophils	Less than 1% (0.02-0.1 × 10^9/L)

Terminologies: Various terminologies used to describe variation in the WBC count are presented in Table 19.2.

Table 19.2: Terminologies used to describe variation in the WBC count

Term	*Definition*	*Example*
Absolute	Actual increase of specific type of WBCs	AEC = Absolute eosinophil count
Relative	Relation of number of specific type of WBCs to total WBC count	If TLC is 6000 cells/cu mm and differential count shows 90% of neutrophils, means neutrophils are increased relatively, because absolute neutrophil count is still normal.
Philia	Indicates affinity or craving. It is used for increased cell count/number of granulocytes	Neutrophilia, eosinophilia, basophilia *Note:* It is also used for hemophilia (clotting factor deficiency), thrombophilia (increased tendency for thrombosis) where there is no increase of cell number.
Cytosis	Indicates more than usual (increased) number of cells.	Leukocytosis, lymphocytosis, monocytosis
Penia	Suffix indicates decreased count/number. Used for any WBC or platelets or non-hematological conditions	Leukopenia, neutropenia, thrombocytopenia, osteopenia (decreased bone mass)

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CLASSIFICATION OF WBC DISORDERS

Major classification of white cell disorders are presented in Fig. 19.2.

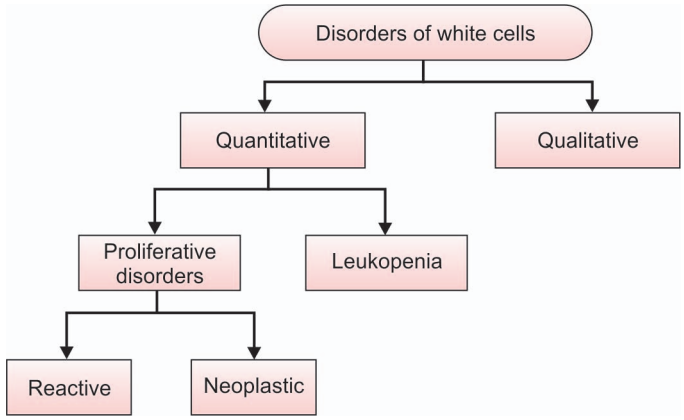


Fig. 19.2: Major classification of disorders of white cells

QUANTITATIVE DISORDERS OF LEUKOCYTES

Quantitative disorders are divided into proliferative disorders and leukopenia. Proliferative disorders include reactive and neoplastic (discussed separately) disorders.

Leukocytosis: It refers to a condition characterized by an increase in the total number of leukocytes in the blood more than 11,000/cu mm (11×10^9/L). Leukocytosis is usually due to increase in the neutrophils, but may also be due to increased lymphocytes (or rarely monocytes and eosinophils).

Causes: Refer Table 41.6.

Leukopenia: It is a condition in which the total leukocyte count is less than 4,000/cu mm (4×10^9/L).

Causes: Refer Table 41.7.

Disorders of Neutrophils

Neutrophils normally have two pools namely circulating pool and marginating pool. Marginating pool consists of neutrophils mobilized at the endothelium of the vessels.

Neutrophilia

Definition: Neutrophilia is defined as an absolute neutrophil count of more than 8000/cu mm (8×10^9/L)

- This term is commonly used when the differential count shows more than 70% neutrophils and is usually accompanied by leukocytosis (15 to 30×10^9/L).

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- In bacterial infections, neutrophils also show *toxic granules* (strongly peroxidase positive), which are coarser and darker than the normal neutrophilic granules. They represent abnormal azurophilic (primary) granules.
- *Döhle bodies* are small, round to oval, grayish blue cytoplasmic inclusions (dilated endoplasmic reticulum) seen in the peripheral part of cytoplasm. They are seen in toxic conditions (e.g. pneumonia, septicemia, burns).

Causes of neutrophilia: Major causes of neutrophilia are shown in Table 19.3.

Table 19.3: Major causes of neutrophilia

1. **Pathological:**
   - Acute bacterial and fungal infections:
     - Localized: Pyogenic microorganisms causing infections, e.g. pneumonias, pyogenic meningitis, cellulitis, diphtheria, abscess, tonsillitis, etc.
     - Generalized: Septicemia, acute rheumatic fever
   - Acute inflammatory processes
     - Inflammatory conditions (acute appendicitis), vasculitis
   - Tissue necrosis
     - Burns
     - Myocardial infarction
     - Gangrene
     - Neoplasms: Tumor necrosis
   - Acute stress or hypoxic states
     - Following hemorrhage, hemolysis and surgery
   - Myeloproliferative neoplasms
     - Chronic myeloid leukemia, polycythemia vera
   - Metabolic
     - Uremia
     - Acidosis
     - Gout
   - Miscellaneous
     - Eclampsia, steroid therapy
2. **Physiological:**
   - Exercise (shift from marginating pool to circulating pool), newborns, extremes of temperature, pain, emotional stress and during obstetric labor

Leukemoid reaction: Sometimes, extreme neutrophilia is associated with the presence of immature white cells in the peripheral blood and is called as ***leukemoid reaction.*** A **leukemoid reaction** is a benign leukocytic proliferation characterized by a total leukocyte count of more than 25×10^9/L with variable number of immature white cells (like band forms, metamyelocytes and myelocytes).

- It should to be differentiated from chronic myelocytic/myeloid leukemia (Table 19.4).

Neutropenia (agranulocytosis)

Definition: Neutropenia is defined as reduction in the absolute neutrophil count (total WBC × % segmented neutrophils and band forms) below 1.5×10^9/L (1500/cu mm).

This usually results in relative increase of lymphocytes. *Agranulocytosis* is the term used when neutrophil count decreases below 0.5×10^9/L and these patients are highly susceptible to bacterial and fungal infections.

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Table 19.4: Differences between leukemoid reaction and chronic myeloid leukemia

	Leukemoid reaction	*Chronic myeloid leukemia*
Clinical features	Features of causative disease	Splenomegaly, lymphadenopathy and bone pain are common
Peripheral blood findings		
WBC		
Total WBC count	Moderately increased, rarely exceeds 50 × 10^9/L	Markedly increased and usually exceeds 50 × 10^9/L
Differential leukocyte count	Shift to the left with few immature forms. Toxic granulation seen.	Shift to the left with numerous immature forms. Myelocyte and neutrophil peak.
Eosinophilia and basophilia	Variable	Present
Leukocyte alkaline phosphatase (LAP)	Increased	Decreased
RBC		
Anemia	Usually minimal or absent	Severe and progressive
Platelets		
Number	Variable	Normal or increased
Extramedullary myeloid tumors	Absent	Present
Philadelphia chromosome	Absent	Present

Etiology: The causes of neutropenia are presented in Table 19.5.

Table 19.5: Causes of neutropenia

1. **Inadequate production:**
   - ***Suppression of stem cells***: In these disorders granulocytopenia represents a component of pancytopenia
     - Aplastic anemia
     - Marrow infiltration
     - Metastatic tumors
     - Granulomatous disorders
   - ***Suppression of committed granulocytic precursors***
     - Drugs and chemicals (e.g. sulfonamides, analgesics, arsenicals)
     - Ionizing radiation
   - ***Diseases associated with ineffective hematopoiesis***
     - Megaloblastic anemias: Vitamin B_{12} or folate deficiency
     - Myelodysplastic syndromes
   - ***Congenital:*** Kostmann syndrome (rare)
   - ***Severe infections***
     - Bacterial (e.g. typhoid, paratyphoid, septicemia)
     - Viral (e.g. influenza, infectious mononucleosis, hepatitis, measles)
     - Rickettsial (e.g. scrub typhus)
     - Protozoal (e.g. malaria, kala-azar)
2. **Increased destruction of neutrophils**
   - ***Immunologically mediated destruction***
     - Idiopathic
     - Secondary
       - Drugs
       - Autoimmune disorders: For example systemic lupus erythematosus
   - **Splenic sequestration** may be associated with pancytopenia

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3. **Shift from the circulating pool to marginating pool:**
   Hemodialysis and cardiopulmonary bypass
4. **Idiopathic:**
   - Hodgkin and non-Hodgkin lymphoma
   - Chronic lymphocytic leukemia
   - Viral infections (HIV, hepatitis)
   - Cyclic neutropenia

Drugs are the major cause of neutropenia which include:

- Chemotherapeutic agents used for cancer treatment may produce agranulocytosis in a predictable and dose-related manner.
- Anti-inflammatory agents (e.g. phenylbutazone ibuprofen): Neutropenia with this group of drugs develops as an idiosyncratic reaction.
- Antibacterial (e.g. chloramphenicol, co-trimoxazole, ciprofloxacin) and antithyroid (e.g. thiouracil, carbimazole) drugs destroy the mature neutrophils by immunological mechanism similar to drug-induced immunohemolytic anemias (refer Chapter 10).
- Phenothiazines (e.g. chlorpromazine): They have a direct toxic effect on granulocytic precursors.

Clinical features of neutropenia

The clinical features of neutropenia are mainly due to bacterial or fungal infections. The infections manifest as sore throat, ulcerating necrotizing lesions within the oral cavity, boils, abscess and skin infections or poor wound healing. The general symptoms usually are fever, chills, malaise, weakness and fatigability. Invasive bacterial or fungal infections may occur in the organs like lungs, urinary tract and kidneys. The patients may develop deep fungal infections due to *Candida* and *Aspergillus*. In severe agranulocytosis, the infection will be severe often terminating in death within hours to few days.

Eosinophilia

Definition: Eosinophilia is defined as an eosinophil count of more than 450/cu mm (0.45×10^9/L). Causes of eosinophilia are presented in Table 19.6.

Basophilia

Normally basophils are less than 1% of WBCs in peripheral blood.

Causes of basophilia are presented in Table 19.7. It may be seen in neoplastic disorders like:

- Chronic myeloid leukemia: Basophilia may be the first sign of blast crisis or accelerated phase.
- Myeloproliferative neoplasms: Persistent basophilia may indicate an unsuspected myeloproliferative neoplasm.
- Acute basophilic leukemia.

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Table 19.6: Causes of eosinophilia

1. **Allergic/atopic conditions**
   - Asthma
   - Urticaria
   - Hay fever
   - Drug reactions
   - Allergic rhinitis
2. **Parasitic infestations (with tissue invasion)**
   - Roundworm infestation
   - Hookworm infestation
   - Filariasis
3. **Fungal infections** (e.g. coccidioidomycosis)
4. **Skin diseases**
   - Dermatitis (eczema)
   - Pemphigus
   - Scabies
   - Dermatitis herpetiformis
5. **Hematological diseases**
   - Chronic myeloid leukemia
   - Polycythemia
   - Hodgkin lymphoma
   - Acute myelomonocytic leukemia
   - Eosinophilic leukemia
6. **Miscellaneous**
   - Tropical eosinophilia
   - Pulmonary eosinophilia
   - *Löeffler's syndrome*
   - Hypereosinophilic syndrome
   - Eosinophilic granuloma

Table 19.7: Causes of basophilia

- Chronic myeloid leukemia
- Immediate hypersensitivity reactions
- Lymphoproliferative disorders
- Mastocytosis
- Post splenectomy
- In few patients with chronic hemolytic anemia
- Viral infections like smallpox, chickenpox

Monocytosis (Table 19.8)

Definition: Monocytosis is defined as more than 10% of differential count or an absolute monocyte count exceeding 500/cu mm (0.5×10^9/L).

Monocytes are increased in recovery stage of chronic infections, chronic inflammatory diseases and few parasitic diseases. In agranulocytosis, its appearance is a favorable sign.

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Table 19.8: Causes of monocytosis

1. **Infections**
   - Bacterial: Tuberculosis, bacterial endocarditis, brucellosis
   - Protozoal: Malaria, kala-azar
   - Spirochetal: Syphilis
   - Rickettsial: Typhus, rocky mountain fever
   - Recovery phase of neutropenia and acute infections
2. **Inflammatory diseases**
   - Inflammatory bowel disease: Ulcerative colitis, Crohn disease
   - Autoimmune diseases: Systemic lupus erythematosus
     Rheumatoid arthritis
   - Sarcoidosis
3. **Hematologic malignancies**
   - Acute monocytic, myelomonocytic and myelocytic leukemias
   - Chronic myelomonocytic leukemia
   - Hodgkin lymphoma
   - Multiple myeloma

Lymphocytosis

Definition: Lymphocytosis is defined as lymphocyte count more than 4,000/cu mm (4×10^9/L)

The term is used when the lymphocytes are more than 45% in the peripheral blood. Common causes of lymphocytosis are given in the Table 19.9

Table 19.9: Causes of lymphocytosis

1. **Acute infections**
   - Viral infections
     Infectious mononucleosis, mumps, measles, chickenpox, infectious hepatitis
   - Toxoplasmosis
2. **Chronic infections/inflammatory diseases**
   - Chronic tuberculosis
   - Syphilis
   - Brucellosis
   - Inflammatory bowel disease: Crohn disease and ulcerative colitis
3. **Hematologic malignancies**
   - Acute lymphoblastic leukemia
   - Chronic lymphocytic leukemia
   - Non-Hodgkin lymphoma with spillover
   - Adult T cell leukemia/lymphoma
   - Hairy cell leukemia

Lymphocytopenia

Definition: Lymphocytopenia is defined as a lymphocyte count below 1,500/cu mm (1.5×10^9/L) in adults and below 3000/cu mm (3×10^9/L) in children.

Absolute reduction in lymphocytes in the peripheral blood is observed following steroid therapy and AIDS. Some of the important causes are listed in the Table 19.10.

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Table 19.10: Causes of lymphocytopenia

1. **Increased destruction**
   - Corticosteroids
   - Cytotoxic drugs
   - Radiation
2. **Decreased production**
   - Aplastic anemia
   - Advanced malignancy: Hodgkin lymphoma
   - Infections- AIDS, miliary tuberculosis
3. **Increased loss via GI tract**
   - Obstruction to intestinal lymphatic drainage (e.g. tumor)
   - Congestive heart failure

CD4 and CD8 Lymphocytes

CD4 and CD8 lymphocyte count is determined by flow cytometry.

Use: In the diagnosis of immune dysfunction (e.g. AIDS).

Decreased CD4

- Acute minor viral infections
- In AIDS severely decreased count is the single best predictor of imminent opportunistic infection.

Plasma Cells

Conditions associated with increased plasma cells are shown in Table 19.11

Table 19.11: Conditions associated with increased plasma cells

Neoplastic

- ***Hematological***
  - Plasma cell leukemia
  - Multiple myeloma
  - Hodgkin lymphoma
  - Chronic lymphocytic leukemia
- ***Non-hematological***
  - Carcinoma liver, prostate, breast

Non-neoplastic

- ***Cirrhosis of liver***
- ***Infections***
  - Bacterial: Tuberculosis
  - Spirochetal: Syphilis
  - Viral: Infectious mononucleosis, measles
  - Parasitic: Malaria

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QUALITATIVE DISORDERS OF LEUKOCYTES

Qualitative disorders of leukocytes (Fig. 19.3) are rare familial disorders which manifest as morphologic changes in the leukocytes. Some of these include:

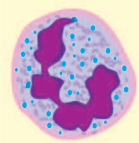
	Mode of inheritance	Characteristic morphology	Appearance of leukocyte	Functional abnormalities
Pelger-Hüet anomaly	Autosomal dominant	Lack of neutrophil nuclear segmentation beyond 2 lobes		Nil
Alder-Reilly anomaly	Autosomal recessive	Large lilac inclusions in cytoplasm of all leukocytes.		Nil
May-Hegglin anomaly	Autosomal dominant	Large basophilic inclusions in all leukocytes. Giant platelets and thrombocytopenia		Abnormal bleeding due to thrombocytopenia
Chediak-Higashi anomaly	Autosomal recessive	Large gray blue granules in cytoplasm of monocytes and granulocytes. Defective lysosomal granules		Poor chemotaxis. increased susceptibility to pyogenic infections. Bleeding tendency
Chronic granulomatous disease of childhood (CGD)	X-linked Autosomal recessive	Normal appearance but defective function		Deficient NADPH oxidase, with absent H_2O_2 production. Phagocytosis and killing of organisms is impaired

Fig. 19.3: Qualitative disorders of leukocytes

- Pelger-Hüet anomaly
- Alder-Reilly anomaly
- May-Hegglin anomaly
- Chediak-Higashi anomaly
- Chronic granulomatous disease of childhood

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SUMMARY

- WBCs include myeloid cells and lymphoid cells.
- The WBC disorders may be quantitative or qualitative.
- Neutrophilia is usually seen in acute bacterial infections, acute inflammation or associated with tissue necrosis. Sometimes, extreme neutrophilia is associated with the presence of immature white cells and known as leukemoid reaction and should be differentiated from leukemia.
- Eosinophilia is usually seen in allergic disorders, worm infestations and variety of skin diseases.
- Lymphocytosis is commonly found in viral infections.

SELF-ASSESSMENT EXERCISES

I. Short Notes

1. Define leukopenia and mention its causes
2. Leukocytosis
3. Neutrophilia
4. Neutropenia
5. Leukemoid reaction
6. Differences between CML and leukemoid reaction
7. Eosinophilia
8. Basophilia
9. Lymphocytosis

II. Multiple Choice Questions

1. **The following statement is NOT true with respect to hypersegmented neutrophils**
   A. They are found in macrocytic anemia
   B. They indicate defect in cytoplasmic maturation
   C. They have six or more than 6 nuclear lobes
   D. They are seen in the peripheral smear of Pernicious anemia
2. **Peripheral blood film in typhoid fever usually shows**
   A. Neutrophilia
   B. Basophilia
   C. Neutropenia
   D. Atypical lymphocytes
3. **All the following conditions usually show leukocytosis EXCEPT**
   A. Abscess
   B. Cellulitis
   C. Enteric fever
   D. Lobar pneumonia

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4. **Eosinophilia may be observed in**
   A. Chickenpox
   B. Typhoid fever
   C. Acute appendicitis
   D. Hodgkin lymphoma
5. **Basophils are increased in**
   A. Allergic disorders
   B. Chronic myeloid leukemia
   C. Bronchopneumonia
   D. Infectious mononucleosis
6. **Lymphocytosis is seen in following conditions EXCEPT**
   A. Whooping cough
   B. *Mycobacterium tuberculosis*
   C. Epstein-Barr virus infection
   D. Staphylococcal infection
7. **The characteristic feature of Pelger Hüet anomaly is**
   A. It shows bilobed neutrophils
   B. It shows lilac inclusions in the cytoplasm of leukocytes
   C. It shows large gray blue granules
   D. It has X-linked mode of transmission

ANSWERS

1. B **2.** C **3.** C **4.** D **5.** B **6.** D **7.** A

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CHAPTER 20

Infectious Mononucleosis

CHAPTER OUTLINE

- Definition
- Pathogenesis
- Clinical Features
- Laboratory Findings
- Lesions Associated with EBV Infection

DEFINITION

Infectious mononucleosis **(glandular fever)** is an **acute, benign, self-limiting** lymphoproliferative disorder **caused by Epstein-Barr Virus** (EBV).

PATHOGENESIS

EBV infects B lymphocytes by binding to the receptor known as **CD21 (CR2)** present on B lymphocyte membrane. CD21 is the receptor for the C3d component of complement. The viral infection begins in the submucosal lymphoid tissues of nasopharynx and oropharynx, particularly the tonsils (Fig. 20.1). The infection of B cells may be either productive (lytic) or latent.

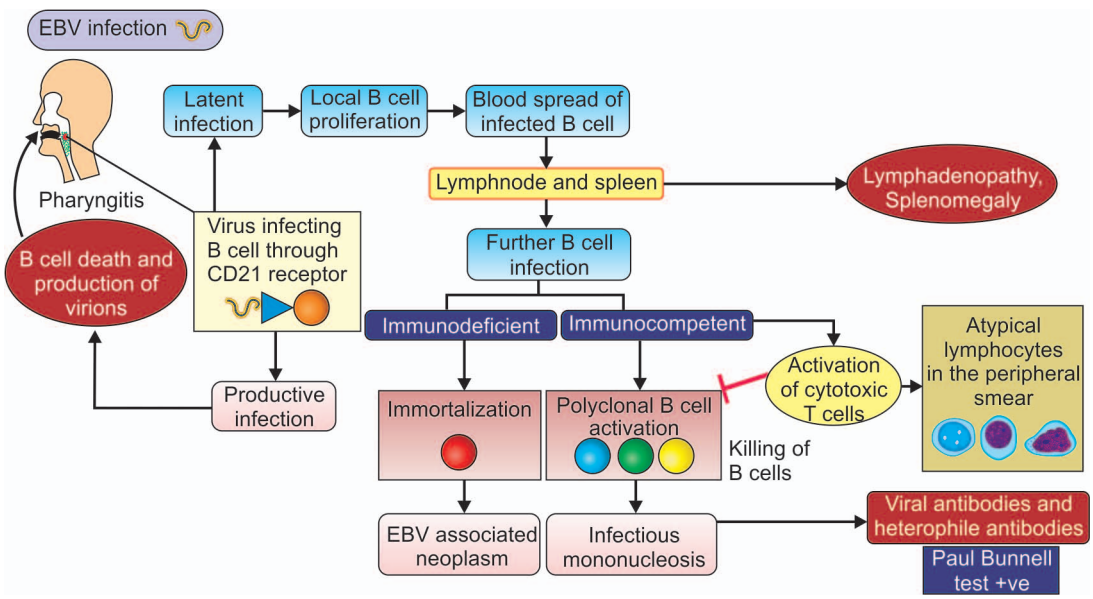


Fig. 20.1: Pathogenesis of EBV associated lesions

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Productive Infection

This type of infection is seen only in a **minority** of B cells. The infected B cells produce and **release virions accompanied by death** of these **B cells.** The released virions in turn infect other B cells.

Latent Infection

In majority cases of EBV infection, the virus becomes latent inside the B cells. There is **no viral replication** and the infected **B cells are not killed.** These latently infected B cells are transformed or **"immortalized"** so that they are capable of **proliferation indefinitely. Immortalization of B cell is the hallmark of EBV infection.**

Molecular Basis of B Cell Proliferation

During latent infection, few EBV genes are expressed which disturb the normal signaling pathways. The EBV gene products include:

EBNA1: This gene product binds the EBV genome to chromosomes of B cells. It mediates episomal persistence and maintenance of EBV.

EBNA2 and latent membrane protein 1 (LMP1): These EBV gene products drive B cell activation and proliferation.

- EBNA2 stimulates transcription of many host cell genes, including genes that drive the cell cycle (e.g. cyclin D).
- LMP1 activates signaling pathways that promote B cell survival and proliferation. This also prevents apoptosis and may act as an oncogene.

Latently infected B cells multiply locally in the pharynx and subsequently circulate throughout the body producing a generalized infection of the lymphoid tissues (e.g. lymphnodes, spleen).

Immune Responses to EBV Infection

EBV induces both humoral and cell mediated immune response.

- ***Humoral immune response:*** EBV induces polyclonal B cell proliferation and the activated B cells produce various antibodies. They are subdivided into:
  - **EB Virus-specific Antibodies:** The most useful EBV-specific antibodies are against:
    - **Viral capsid antigens (VCAs)**
    - **EBV Nuclear Antigen (EBNA)**

    Both VCA and EBNA antibodies are initially of IgM type followed by IgG type.
  - **Heterophile Antibodies**
    - *Hetero* and *phile* are derived from the Greek words meaning 'different' and 'affinity', respectively. An antibody formed in response to an antigen from one species but cross-reacts against antigens on the cells of other species is termed as **heterophile antibody.** These are produced against poorly defined antigens and are **nonspecific.**

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  - Heterophile antibodies found in patients with infectious mononucleosis are capable of **agglutinating sheep/ox/horse red cells** but **not the human red cells.** This is the basis of **Paul Bunnell test** and other screening tests. They do not react with EBV antigens.
  - **Autoantibodies:** These are responsible for positive Wassermann reaction.
- ***Cell mediated immune response:*** Activated B cells also stimulate cell mediated immune response and activate CD8+ cytotoxic T cells and NK cells. The latter two cells (i.e. CD8+ cytotoxic T cells and NK cells) are **responsible for killing of the viral infected B cells** and also inhibit production of immunoglobulins by B cells. Though EBV infects B cells, the characteristic **atypical lymphocytes** seen in the peripheral blood are mainly **CD8+ cytotoxic T cells and NK cells.** The proliferation of T cells in lymphoid tissues is responsible for lymphadenopathy and splenomegaly.

In immunocompetent individuals, the infection may either remain asymptomatic or develop self-limited infectious mononucleosis. In immunodeficient persons, infected B cells can progress to uncontrolled proliferation causing EBV-associated B cell neoplasms.

CLINICAL FEATURES

- ***Age:*** It is usually seen in young adults among upper socioeconomic classes in developed nations and children of low socio-economic status.
- ***Mode of transmission:*** It is transmitted by kissing (hence the nickname kissing disease) through saliva.
- ***Incubation period:*** It ranges from 4 to 8 weeks.
- ***Signs and symptoms:*** Infectious mononucleosis usually presents with nonspecific prodromal symptoms followed by the **classical triad** of fever, pharyngitis and lymphadenopathy.
  - **Fever:** It is associated with headache, malaise, nausea, anorexia and myalgia.
  - **Pharyngitis:** Sore throat occurs in most of the patients.
  - **Lymphadenopathy:** Usual lymphnodes enlarged are cervical, axillary and inguinal. Mild to moderate *splenomegaly* is observed in about 50% of the patients.

LABORATORY FINDINGS

Diagnosis of IM can be done with following findings:

Peripheral Smear

The smear findings provide important clues to the diagnosis and can be confirmed by serological tests.

- **RBCs:** Show normocytic normochromic picture.
- **WBCs:** The **total leukocyte count** is usually **increased** (12,000 to 25,000 cells/cu mm) and is due to **absolute lymphocytosis**, which constitutes more than 50% of the leukocytes.

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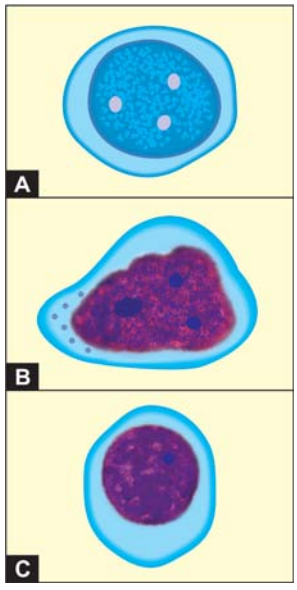
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The characteristic finding is the presence of transformed lymphocytes in the smear. These cells, known as virocytes/**atypical** (activated) **lymphocytes (mononuclear cells)**; hence the disease is termed as infectious mononucleosis. These atypical lymphocytes are **not pathognomonic** and are also observed in other viral fevers. These atypical lymphocytes represent transformed polyclonal **CD8+ subset (cytotoxic) of T cells** and not the virus-infected B cells.

Atypical features which help to identify them from normal lymphocytes include:

- *Size and shape:* Large in size (12-16 μm in diameter) and irregular in shape.
- *Cytoplasm:*
  - Abundant with scattered azurophilic granules
  - Increased cytoplasmic basophilia, more prominent at the edges
  - Cytoplasmic vacuoles and the cytoplasm shows scalloped margins at points of contact with other cells.
- *Nucleus:*
  - Oval, indented or folded nucleus
  - Two to three times larger than those of normal lymphocytes
  - Diffuse chromatin pattern with prominent nucleoli.

Types of atypical lymphocytes: Morphologically they are mainly of three types: blastoid, monocytoid and plasmacytoid. Earlier these have been designated as Downey type 1, 2, 3 cells (Figs 20.2 and 20.3).



Figs 20.2A to C: Three types of atypical lymphocytes, namely: A. Blastoid, B. Monocytoid, and C. Plasmacytoid

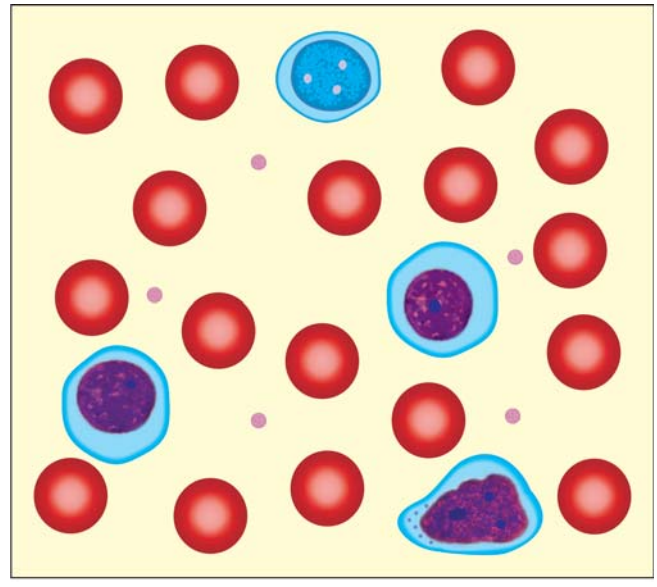


Fig. 20.3: Peripheral blood smear in infectious mononucleosis with 'atypical' lymphocytes

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- **Blastoid (Downey type 1):** These cells have open reticular nuclear chromatin with thin rim of cytoplasm and should be differentiated from blast cells.
- **Monocytoid (Downey type 2):** The cells contain a kidney shaped/lobulated nucleus with relatively open chromatin and vacuolated cytoplasm.
- **Plasmacytoid cells (Downey type 3):** The cells resemble plasma cells and show chromatin condensation (cartwheel pattern), perinuclear hof with basophilic cytoplasm.

- **Platelets:** Normal or mildly decreased.

Serological Tests

- **Demonstration of heterophile antibodies:** The following tests will be useful for demonstration of heterophile antibodies
  - Paul Bunnell test is characteristically positive.
  - Monospot test is a sensitive slide test.
- **Demonstration specific antibodies against EBV antigens:** These are demonstrated by ELISA. The EBV specific antibodies are:
  - Antibody against viral capsid antigens (anti-VCA): These antibodies are initially of IgM type and later of IgG type (which persists for life).
  - Antibodies to Epstein-Barr nuclear antigen (EBNA): This can be demonstrated by polymerase chain reaction (PCR).

Lymph Nodes

They are discrete and are enlarged throughout the body. Microscopically, the paracortical regions are expanded due to proliferation of activated T cells.

LESIONS ASSOCIATED WITH EBV INFECTION

These are shown in Table 20.1.

Table 20.1: Lesions associated with EBV infection

- Infectious mononucleosis
- B cell neoplasms
  - African Burkitt lymphoma
  - B cell lymphoma in immunosuppressed persons
- Hodgkin lymphoma: Lymphocyte depletion (90% of cases), mixed cellularity (70%) and lymphocyte-rich (40%)
- Extranodal NK/T cell Lymphoma
- Nasopharyngeal carcinoma, some cases of gastric carcinomas, thymic carcinoma.

SUMMARY

- IM is caused by Epstein-Barr virus and usually transmitted via saliva.
- IM is characterized by fever, lymphadenopathy and peripheral smear with atypical lymphocytes.

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- Paul Bunnell test is positive and is due to heterophile antibodies.
- EBV can also cause B cell neoplasms, Hodgkin lymphoma and nasopharyngeal carcinoma.

SELF-ASSESSMENT EXERCISES

I. Short Notes

1. Infectious mononucleosis
2. Laboratory findings in infectious mononucleosis
3. Lesions associated with EBV infection
4. Atypical lymphocytes

II. Multiple Choice Questions

1. **The hallmark of latent infection by EBV is**
   A. Immortalization of B cells
   B. Immortalization of T cells
   C. Apoptosis of B cells
   D. Apoptosis of T cells
2. **Heterophile antibody is demonstrated by**
   A. Ham's test
   B. Widal test
   C. Rh antigen
   D. Paul Bunnell test
3. **The characteristic atypical lymphocytes seen in the peripheral blood of patients with infectious mononucleosis are**
   A. Monocytes
   B. B lymphocytes
   C. CD4+ T lymphocytes
   D. CD8+ cytotoxic T cells
4. **All the following conditions are associated with EBV infection EXCEPT**
   A. Chronic myeloid leukemia
   B. Infectious mononucleosis
   C. African Burkitt lymphoma
   D. Nasopharyngeal carcinoma

ANSWERS

1. A **2.** D **3.** D **4.** A

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Chapter 21 Introduction to Acute Leukemia

Chapter Outline

- Neoplastic Proliferations of White Cells
- Acute Leukemia

NEOPLASTIC PROLIFERATIONS OF WHITE CELLS

Malignant neoplasms of white cells, though less frequent than the reactive proliferations, are the most important from clinical point of view. They can be categorized into three major groups (Table 21.1).

Table 21.1: Major categories of neoplastic proliferation of white cells

Myeloid neoplasms
- Acute myeloid leukemias
- Myelodysplastic syndromes
- Myeloproliferative neoplasms
- Myelodysplastic/myeloproliferative neoplasms

Lymphoid neoplasms
- Lymphocytic/lymphoid leukemias
  - Acute lymphoblastic leukemias
  - Chronic lymphocytic leukemias
- Lymphoma: Non-Hodgkin and Hodgkin lymphoma

Histiocytic and dendritic cell neoplasms
- Langerhans cell histiocytosis

Myeloid Neoplasms

They form a heterogeneous group of neoplasms, which arise from early hematopoietic progenitor cells. These neoplasms primarily involve the bone marrow and to a lesser extent the secondary hematopoietic organs (the spleen, liver, and lymph nodes). They usually present with symptoms related to altered hematopoiesis. They can be broadly categorized as:

- **Acute myeloid leukemias:** They are characterized by accumulation of immature progenitor cells in the bone marrow, which suppresses the normal hematopoiesis.
- **Myelodysplastic syndromes (MDS):** These syndromes are associated with ineffective hematopoiesis which leads to peripheral blood cytopenias.

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- **Myeloproliferative neoplasms (MPN):** The characteristic feature of these neoplasms is increased production of one or more terminally differentiated myeloid elements. They usually produce elevated peripheral blood counts.
- **Myelodysplastic/myeloproliferative neoplasms:** These neoplasms present the clinical, laboratory and morphological features that overlap both MDS and MPN.

Lymphoid Neoplasms

These include a diverse group of neoplasms of B cell, T cell, and NK cell origin. They can be sub divided into two categories.

- **Lymphocytic/lymphoid leukemias:** These neoplasms present with widespread involvement of the bone marrow and usually (but not always) the peripheral blood.
- **Lymphoma:** They represent lymphoid proliferations which present as tissue masses. These are categorized into two main groups namely: Non-Hodgkin and Hodgkin lymphoma.

However this division is only for better understanding, because some lymphomas occasionally may present and/evolve as leukemias. Conversely, leukemias may sometimes appear as soft-tissue masses unaccompanied by bone marrow involvement.

Histiocytic and Dendritic Cell Neoplasms

These are uncommon proliferative lesions of macrophages and dendritic cells. Langerhans cell is a special type of immature dendritic cell which can produce a spectrum of neoplastic disorders known as *Langerhans cell histiocytosis.*

Both myeloid and lymphoid neoplasms can present as acute and chronic leukemias. Though acute lymphoid and myeloid leukemias are distinct (immunophenotypically and genotypically), they usually have certain features in common. Hence, the following discussion will describe the common features of acute leukemias.

ACUTE LEUKEMIA

Definition

Leukemia is defined as a group of malignant stem cell neoplasms characterized by:

- Diffuse replacement of bone marrow with proliferating leukocyte precursors (blast cells)
- Abnormal numbers and forms of immature white blood cells in circulation.
- In acute leukemia the immature, neoplastic leukemic 'blast' cells proliferate and accumulate, but fail to mature. The blasts diffusely replace the normal bone marrow and a variable number of these accumulate in the peripheral blood.
- Acute leukemia should be diagnosed when the blast cells constitute more than 20% of the nucleated cells in the marrow (normally blast cells are less than 5%).

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- In some patients there may be very few /no blasts in the peripheral blood and is termed as **aleukemic/subleukemic leukemia.**
- Infiltration of the marrow by leukemic cells finally results in bone marrow failure resulting in cytopenias. Most patients present with consequences of cytopenias (anemia, neutropenia and thrombocytopenia) particularly in the acute leukemias.

Etiology and Pathogenesis

In the majority of acute leukemias the cause is not known. Numerous risk factors may cause mutations in the genes involved in regulating cell proliferation and differentiation. These genes include oncogenes and tumor suppressor genes.

Risk Factors

Environmental factors

- **Ionizing radiation:** Exposure to ionizing radiation and X-rays are associated with increased risk of leukemias. The evidences for this association are:
  - Survivors of atomic bomb explosions in Hiroshima and Nagasaki, who had high incidence of acute myelogenous leukemia (AML) and chronic myeloid leukemia (CML).
  - Increased risk of AML (secondary) in patients with malignancies/ neoplasms treated by radiation.
- **Drugs**: Drugs can cause secondary hematopoietic neoplasms.
  - Leukemia develops in patients who have been administered alkylating agents for neoplasms like Hodgkin lymphoma. The various drugs include nitrogen mustard, chlorambucil, etc.
  - AML occurs in myeloma patients treated with melphalan.
  - Leukemia follows chemotherapy of lung and ovarian cancer. Some anticancer drugs induce myelodysplastic changes with certain chromosomal abnormalities and subsequently develop AML.
- **Chemicals:** Benzene (used in paint industry, plastic glues, etc) causes chromosomal abnormalities resulting in higher incidence of acute leukemia, myelodysplastic syndrome and aplastic anemia.

Genetic disorders

A few genetic disorders may be associated with acute leukemias, e.g. Down syndrome (ALL or AML), Fanconi anemia (AML), ataxia telangiectasia (ALL, NHL).

Acquired Disorders

- Acquired stem cell disorders like PNH and aplastic anemia may transform into acute leukemia.
- AML may develop de novo or secondary to myelodysplastic syndrome (MDS).

Classification

Depending on microscopic appearance of the involved cell and the course of leukemias, they were initially classified as:

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- **Acute**
  - **Acute myelogenous/myeloblastic/myelocytic/myeloid leukemia (AML)**
  - **Acute lymphoblastic/lymphocytic leukemia (ALL)**
- **Chronic**
  - **Chronic myeloid leukemia (CML)**
  - **Chronic lymphocytic leukemia (CLL)**

Differentiation of two broad groups of ALL and AML from each other is important, because of difference in the treatment, course and prognosis.

FAB Classification of Acute Leukemias

According to FAB, the marrow should show a blast count of 30% or more for the diagnosis of acute leukemia.

- **French, American and British (FAB)** classification was formulated in 1976 for classification of acute leukemias. This was based on the ***morphological and cytochemical characteristics of blast cells.***
- Subsequently, classification has been revised from time to time with inclusion of few new entities. **Revised FAB classification** (Tables 21.2 and 21.3) included parameters which affect prognosis. Thus, it included not only morphology and cytochemistry but also immunophenotyping, cytogenetics and molecular genetics.

Table 21.2: Revised FAB Classification of acute myelogenous leukemias (AML)

Type	*Marrow morphology/comments*
M0 Minimally differentiated AML	Blasts lack morphologic and cytochemical markers of myeloblasts. Express myeloid lineage antigens.
M1 AML without differentiation	Blasts more than 30% and peroxidase positive. Less than 10% promyelocytes and maturing myeloid forms. Rare Auer rods.
M2 AML with maturation	Blasts more than 30% and peroxidase positive. More than 10% promyelocytes and maturing myeloid forms. Few Auer rods.
M3 Acute promyelocytic leukemia	Hypergranular promyelocytes with many Auer rods (Faggot cells) M3v-hypo (micro) granular variant.
M4 Acute myelomonocytic leukemia	Myelocytic and monocytic differentiation evident. M4 with eosinophilia (M4eo)
M5 Acute monocytic leukemia	Predominantly monoblasts and promonocytes seen
M6 Acute erythroleukemia	Dysplastic erythroid precursors seen along with myeloblasts
M7 Acute megakaryocytic leukemia	Blasts of megakaryocytic lineage predominate

Table 21.3: FAB classification of acute lymphoid leukemias (ALL)

FAB type	*L1*	*L2*	*L3*
Cell size	Small cell size	Large heterogeneous cell population	Large, homogeneous cell population
Nuclear Characteristics			
Shape	Regular	Irregular, clefting and indentation common	Regular, oval or round
Chromatin	Condensed	Dispersed chromatin	Finely stippled
Nucleolus	Small and inconspicuous	Visible, 1-2 in number	Usually prominent

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Cytoplasmic characteristics			
Amount	Scanty	Variable, often abundant	Moderately abundant
Cytoplasmic basophilia	Slight to moderate	Variable	Strong
Cytoplasmic vacuolation	Absent	Variable	Prominent and oil red O stain positive

Revised WHO Classification (2008) of Acute Myelogenous Leukemia

- Revised WHO classification (Table 21.3) of acute leukemia incorporates parameters which include morphology, cytochemistry, immunophenotyping, cytogenetics, molecular genetics (which are related to prognosis) and clinical features (Table 21.4).
- The number of **blasts** necessary for the diagnosis is **more than 20%** in bone marrow when compared to 30% in FAB classification.
- AML is classified into seven major categories (Table 21.4).

Table 21.4: WHO Classification (2008) of acute myeloid leukemia and related precursor neoplasms

Class	*FAB category*	*Prognosis*
I. AML WITH RECURRENT GENETIC ABNORMALITIES		
AML with t(8;21)(q22;q22); *RUNX1-RUNX1T1*	M2	Favorable
AML with inv(16)(p13;1q22); *CBFB-MYH11*	M4eo	Favorable
APL with t(15;17)(q22;q12); *PML-RARA*	M3, M3v	Intermediate
AML with t(9;11)(p22;q23); *MLLT3-MLL*	M4, M5	Intermediate
AML with t(6;9)(p23;q34); *DEK-NUP214*	–	Poor
AML with inv(3)(q21q26.2) *RPN1-EVI1*	–	Poor
AML(megakaryoblastic) with t(1;22) (p13;q13); *RBM15-MKL1*	–	Variable
AML with mutated *NPM1*		Favorable
AML with mutated *CEBPA*	Variable	Favorable
II. AML WITH MDS-RELATED CHANGES	Variable	Poor
III. THERAPY-RELATED MYELOID NEOPLASMS	Variable	Poor
IV. AML NOT OTHERWISE SPECIFIED		
AML minimally differentiated	M0	Intermediate
AML without maturation	M1	Intermediate
AML with maturation	M2	Intermediate
Acute myelomonocytic leukemia	M4	Intermediate
Acute monoblastic and monocytic leukemia	M5a, M5b	Intermediate
Acute erythroid leukemia	M6a, M6b	Intermediate
Acute megakaryoblastic leukemia	M7	Intermediate
Acute basophilic leukemia	–	Poor
Acute panmyelosis with myelofibrosis	–	Poor
V. MYELOID SARCOMA	–	–
VI. MYELOID PROLIFERATION RELATED TO DOWN SYNDROME		
Transient abnormal myelopoiesis	–	–
Myeloid leukemia associated with Down syndrome	–	Variable
VII. BLASTIC PLASMACYTOID DENDRITIC CELL NEOPLASM	–	Poor

AML = Acute myeloid leukemia; APL = Acute promyelocytic leukemia; MDS = Myelodysplastic syndrome

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Present WHO classification considers acute lymphoblastic leukemias as precursor-B/ T lymphoblastic leukemia/lymphoma (Refer Chapter 30).

Diagnosis of Acute Leukemia

Presently the diagnosis of acute leukemia requires the presence of 20% or more blasts in the bone marrow (WHO criteria). The sub classification of acute leukemia is based on: (1) morphology, (2) cytochemistry, (3) immunophenotyping, (4) cytogenetics (e.g. karyotyping) and (5) molecular genetics.

Morphology of the Blasts

Morphology of the blasts in peripheral blood and bone marrow is the most important feature. Blast cells have following morphological features:

- High N:C ratio
- Nucleus is large with open chromatin and 1 to 5 nucleoli.
- Thin rim to moderate amount of cytoplasm.

Cytochemistry

- Cytochemistry is the study of chemical elements found in the cytoplasm of the cells.
- Sometimes differentiating AML from ALL (Table 21.5 and Fig. 21.1) is not possible only on morphological grounds. In such cases cytochemical stains (Figs 21.2A to C) will be of help (Chapter 47).

Table 21.5: Differences between myeloblast and lymphoblast based on morphology and cytochemistry

	Lymphoblast	*Myeloblast*
Size	2-3 times the size of lymphocyte	3-5 times the size of lymphocyte
Cytoplasmic characters		
Amount	Scanty (less cytoplasm than myeloblast)	Scanty to moderate (more cytoplasm than lymphoblast)
Color	Blue	Gray
Cytoplasmic granules	Agranular	May have cytoplasmic granules
Auer rod	–ve	+ve
Nuclear Characters		
Nuclear chromatin	Uniform, coarse	Uniform, fine
Nucleoli	Inconspicuous or 1 to 2	3 to 5, prominent
N:C Ratio	High	High
Accompanying cells	Lymphocytes	Promyelocytes, myelocytes, metamyelocytes, band forms and neutrophils
Cytochemistry		
Myeloperoxidase	–ve	+ve
Sudan Black	–ve	+ve
PAS	Block positivity	–ve
Non-specific esterase	–ve	+ve in M4 and M5

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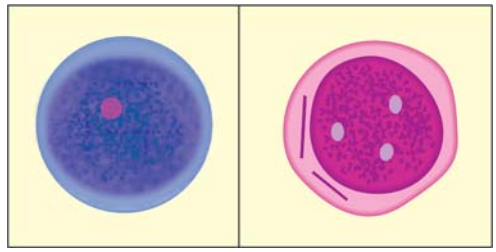
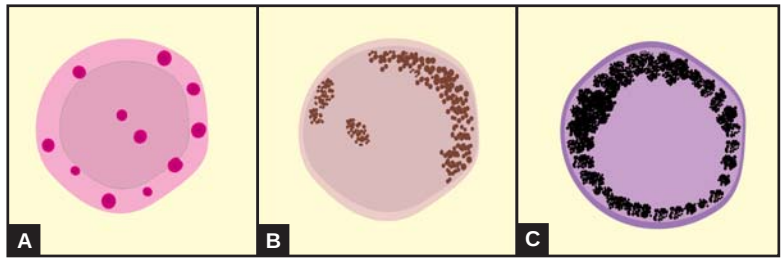


Fig. 21.1: Lymphoblast (left) versus myeloblast (right)



Figs 21.2A to C: Cytochemistry of blasts in acute leukemia: A. Periodic acid Schiff (PAS) stain showing lymphoblast with block positivity, B and C. Myeloblast stained positively with myeloperoxidase and Sudan black respectively

Immunophenotyping (Table 21.6)

The leukemic cells express cell surface and cytoplasmic antigens which can be detected by using monoclonal antibodies (MCAs) directed against those antigens. Immunophenotyping is useful when morphological and cytochemical features cannot differentiate the type of leukemia.

Uses:

- To distinguish ALL from AML
- To distinguish B or T lineage in ALL

Immunophenotyping can be performed by two techniques.

- *Immunocytochemistry:* By this method, peripheral smear and/or bone marrow smears are stained by MCAs.
- *Flow cytometry*

Table 21.6: Useful panel of monoclonal antibodies (MCAs) for differentiating AML from ALL

Type of leukemia	*Myeloid cell antigens*		*B lymphoid cell antigens*		*T Lymphoid cell antigens*	
	CD13	***CD33***	***CD10***	***CD19***	***CD22***	***CD2, CD3, CD5, CD7***
AML	+	+	–	–	–	–
ALL-B lineage	–	–	+	+	+	–
ALL-T lineage	–	–	+/–	–	–	+

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Cytogenetics

- Cytogenetics is defined as the study of chromosomes, their structure and their inheritance. One of the ways of studying cytogenetics is by karyotyping.
- Chromosomal abnormalities may be classified as structural or numerical.
  - **Numeric abnormalities:** They are often subclassified as aneuploidy and polyploidy.
    - **Aneuploidy** is characterized by number of chromosomes which is not a multiple of the haploid number of 23 chromosomes. The examples are: trisomy (with extra-chromosome), monosomy (absence of single chromosome).
    - **Polyploidy** is characterized by chromosome number more than 46, but usually shows exact multiples of haploid chromosome number 23. Example a karyotype with 69 chromosomes (triploidy) and with 92 chromosomes (tetraploidy).
  - **Structural abnormalities:** These include balanced and unbalanced rearrangements, inversions, deletions, insertions, isochromosomes, ring formations, translocations and duplications.
- Cytogenetic analysis is important for determination of prognosis and selection of post remission therapy. WHO has incorporated cytogenetic abnormalities in the present classification (2008) of acute leukemias classification.

Molecular Genetics

Molecular genetics is based on DNA analysis and its usefulness in acute leukemias is to:
- Determine the clonal origin.
- Detect T cell receptor (TCR) genes in ALL.

Methods:
- Fluorescent *in situ* hybridization (FISH)
- Southern blot technique
- Polymerase chain reaction

Clinical Presentation of Acute Leukemia

Though ALL and AML are distinct (immunophenotypically and genotypically), they usually have similar clinical presentation (Table 21.7).

Table 21.7: Differences in the clinical presentation between ALL and AML

Clinical features	*ALL*	*AML*
Age	Predominantly children	Predominantly adults
Leukostasis	Rare	Common
Coagulopathy (DIC)	Absent	Seen in M3
Gingival hypertrophy and dermal infiltrate	Absent	Seen in M4 and M5
Hepatosplenomegaly	Occasional	Frequent
Lymphadenopathy	Occasional	Rare
Leukemic meningitis	Common	Rare

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- ***Bone marrow failure*:** Most often leukemic patients present with signs and symptoms of bone marrow failure due to replacement of normal marrow hematopoietic cells by leukemic blast cells. All or any of the three cell lines are decreased, which results in anemia, neutropenia and thrombocytopenia.
  - Anemia causes fatigue, weakness and hypermetabolic state which is directly related to the degree of anemia.
  - Neutropenia results in life-threatening infections by bacteria or opportunistic fungi, *Pseudomonas* and commensals. The infection may develop in the oral cavity, skin, lungs, kidneys, urinary bladder and colon. The common presentations include respiratory infections (pneumonia), cellulitis or sepsis.
  - Thrombocytopenia presents as bleeding manifestations in the form of petechiae, atraumatic ecchymoses gum bleeding, epistaxis, urinary tract and fundal hemorrhages.
  - Marrow expansion and infiltration of the subperiosteum causes bone pain (more common in ALL) and sternal tenderness.
- ***Leukostasis*:** Stasis of blood flow may develop when the blast count in the peripheral blood is above 50×10^9 (50,000/cu mm). Leukostasis is more common in AML than ALL because myeloblast is larger and expresses adhesive proteins. Most of the patients have leukocytosis and is responsible for the following symptoms:
  - Cerebral leukostasis may cause headache, confusion and visual disturbances.
  - Pulmonary leukostasis can cause dyspnea at rest and tachypnea.
- ***Coagulopathy:*** Both disseminated intravascular coagulation (DIC) and primary fibrinolysis may lead to hemorrhagic diathesis in addition to that due to thrombocytopenia. DIC is observed in AML-M_3 (promyelocytic leukemia).
- ***Extramedullary infiltration:*** Leukemic blasts may infiltrate organs like lymphnodes, spleen, gingiva, skin (leukemia cutis) and meninges.
  - **Gingival hypertrophy and infiltration of skin (leukemia cutis)*:*** They may be found in monocytic type of AML namely AML-M_5 and AML-M_4. This may be due to the normal tendency of monocytes to extravasate into tissues.
  - **Hepatosplenomegaly:** Splenomegaly and hepatomegaly may be found and is more common in AML.
  - **Lymphadenopathy:** It is found occasionally in ALL and is rare in AML.
  - **Leukemic meningitis:** It is rare and less common than in ALL.
- ***Tumor lysis syndrome (TLS):*** It is a group of metabolic complications that can occur after treatment of leukemias (also in lymphomas) and is caused by the breakdown products of dying tumor cells. The killed tumor cells release intracellular ions and large amounts metabolic byproducts (includes potassium, phosphate and nucleic acids) into the systemic circulation. They result in metabolic abnormalities like hyperkalemia, hyperphosphatemia, hyperuricemia, hyperuricosuria, hypocalcemia, and consequent acute uric acid nephropathy and renal failure.

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SUMMARY

- Leukemia is a stem cell neoplasm characterized by diffuse replacement of bone marrow with blast cells and immature white blood cells in circulation.
- Older classification is the FAB classification. The present one is the WHO classification which requires presence of 20% or more blasts for the diagnosis of acute leukemia.
- The subclassification of leukemia is based on cellular morphology, cytochemistry, immunophenotyping, molecular genetics and cytogenetics.
- The clinical features are mainly due to bone marrow failure, leukostasis and coagulopathies.

SELF-ASSESSMENT EXERCISES

I. Short Notes

1. Define and classify leukemia
2. Mention the FAB and WHO classification of acute myelogenous leukemia
3. FAB classification of acute lymphoid leukemias
4. Etiology of leukemia
5. Differences between lymphoblast and myeloblast
6. Differences between L1, L2 and L3 subtypes of ALL
7. Differences between cytochemistry of lymphoblast and myeloblast
8. Useful panel of monoclonal antibodies for differentiating AML from ALL
9. Differences in the clinical presentation between ALL and AML

II. Multiple Choice Questions

1. **Risk factors that predispose to leukemia include all EXCEPT**
   A. Down syndrome
   B. Ionizing radiation
   C. Infectious mononucleosis
   D. Myelodysplastic syndrome
2. **The syndrome which has an increased predisposition for leukemia is**
   A. Klinefelter syndrome
   B. Down syndrome
   C. Turner syndrome
   D. Peutz Jeghers syndrome
3. **Revised FAB classification of AML is based on**
   A. Morphological features
   B. Morphological and cytochemical characteristics
   C. Morphological, cytochemical characteristics and cytogenetic abnormalities
   D. Morphological, cytochemical characteristics and molecular genetic abnormalities

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4. **The following is NOT a feature of blast cells observed in leukemias**
   A. High N:C ratio
   B. Large pleomorphic nuclei
   C. Nucleus with open chromatin and 1 to 5 nucleoli
   D. Thin rim to moderate amount of cytoplasm
5. **The cytochemical stain which distinguishes myeloblast from lymphoblast is**
   A. TRAP
   B. Myeloesterase
   C. Myeloperoxidase
   D. Leukocyte Alkaline Phosphatase
6. **The cytochemical stain which shows block positivity in lymphoblast is**
   A. PAS
   B. Sudan black
   C. Nonspecific esterase
   D. Myeloperoxidase
7. **The monoclonal antibodies which show positivity in AML is**
   A. CD13 and CD33
   B. CD10, CD19 and CD22
   C. CD2 and CD3
   D. CD5 and CD7
8. **The monoclonal antibodies which show positivity in B-lineage ALL is**
   A. CD2 and CD3
   B. CD5 and CD7
   C. CD13 and CD33
   D. CD10, CD19 and CD22
9. **The following is NOT a common presenting feature observed in AML**
   A. Leukemic meningitis common
   B. Hepatosplenomegaly frequent
   C. Leukostasis common
   D. Predominantly seen in adults
10. **The AML subtype presenting with gingival hypertrophy and leukemia cutis is**
    A. M1
    B. M2
    C. M3
    D. M4
11. **The translocation found in AML FAB type M3v is**
    A. t(8;14)
    B. t(8;21)
    C. t(9;22)
    D. t(15;17)

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12. According to WHO, the minimum percentage of myeloid blasts in bone marrow necessary for the diagnosis of AML is

A. 10%
B. 20%
C. 30%
D. 40%

13. In FAB classification of AML, M6 stands for

A. Erythroleukemia
B. Monoblastic leukemia
C. Myelomonocytic leukemia
D. Promyelocytic leukemia

14. The cells associated with formation of chloroma are

A. Plasmablasts
B. Immunoblasts
C. Myeloblasts
D. Lymphoblasts

ANSWERS

1. C	**2.** B	**3.** B	**4.** B	**5.** C	**6.** A	**7.** A
8. D	**9.** A	**10.** D	**11.** D	**12.** B	**13.** A	**14.** C

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Chapter 22 Acute Lymphoblastic Leukemia

Chapter Outline

- ❖ Acute Lymphoblastic Leukemia
- ❖ Acute Lymphoblastic Lymphoma

ACUTE LYMPHOBLASTIC LEUKEMIA

Definition

Acute lymphoblastic leukemia/lymphoma (ALL) is a group of neoplasms consisting of immature, precursor B (pre-B) or T (pre-T) lymphocytes known as **lymphoblasts.** Morphologically it is difficult to distinguish malignant pre-B and pre-T lymphoblasts. It requires immunophenotyping for subclassification of ALL.

Leukemia vs. Lymphoma:

- **Leukemia:** Proliferations of lymphoblasts in the bone marrow and peripheral blood are termed as lymphoblastic leukemia.
- **Lymphoma:** Primary involvement of lymphnodes or extranodal sites by lymphoblasts is referred to as lymphoblastic lymphoma.

About 85% of ALLs are tumors of **precursor B cells** which are primarily seen in childhood and present as acute leukemias. Remaining 15% of ALLs are derived from **precursor T cells** and present in adolescent males as **lymphomas**, often with involvement of mediastinum (thymus).

Molecular Pathogenesis

Leukemia, like any cancer, is a multistep process and single mutations are not enough to produce ALL.

- Numerical or structural chromosomal abnormalities are found in about 90% of ALLs.
  - **Numerical abnormality:** Most common is hyperploidy (>50 chromosomes) and hypoploidy.
  - **Structural abnormality:** Balanced chromosomal translocations including the Philadelphia chromosome are also seen. Most chromosomal abnormalities dysregulate the expression and function of transcription factors involved in development of normal B and T cells.

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- Most T-ALLs have mutations in NOTCH1 (gene required for T cell development).
- Most B-ALLs have mutations in genes PAX5, E2A and EBF (required for B cell development) or a balanced translocation t(12;21) involving the genes TEL and AML1.
- These mutations result in proliferation without maturation.

Classification of Acute Lymphoblastic Leukemia (see Tables 21.3 and 30.1)

Clinical Features

Age and Sex: ALL is the most common hematological malignancy of children. Most commonly found between 1 to 5 years of age and second peak in adults is found between 30 to 40 years. There is a slight male preponderance.

Onset: Abrupt.

Symptoms: They are related to depressed marrow function due to infiltration by blasts. This includes symptoms due to anemia, neutropenia and thrombocytopenia. In addition to general features of acute leukemia (Refer pages 165-166) ALL may present with:

- **Lymphadenopathy:** It is present in 75% of patients.
- **Hepatosplenomegaly:** Splenomegaly is more common than hepatomegaly.
- **Mediastinal mass:** Mediastinal thymic mass may compress blood vessels and airways in the mediastinum. This is more common in T-ALL.
- **Central nervous system involvement:** It may spread into the meninges and is more common in ALL (pre-B).
- **Testicular enlargement**.

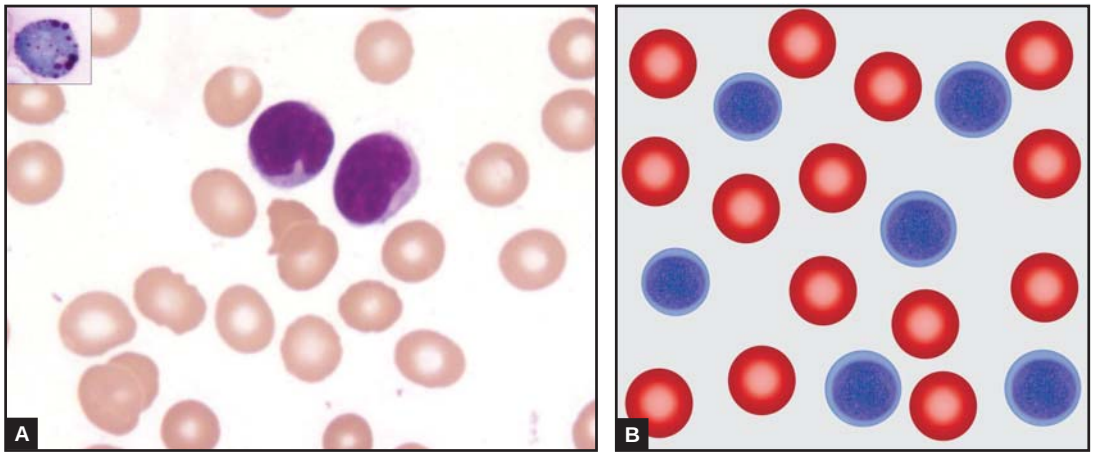
Laboratory Findings

Peripheral Blood

- **Total WBC count:** Total white cell count is markedly raised ranging from 20×10^9/L to 200×10^9/L. In few cases count may be normal or even reduced.
  - ***Subleukemic leukemia:*** It is characterized by a total white cell count lower than 4×10^9/L and peripheral blood shows very few blasts.
  - ***Aleukemic leukemia:*** The term aleukemic leukemia is used when total white cell count is low ($< 4 \times 10^9$/L) with no blasts in the peripheral blood.
    The diagnosis in such cases requires bone marrow examination to demonstrate 20% or more blast cells.
- **Hemoglobin:** Hemoglobin progressively decreases and may be reduced even to 3 gm/dL.
- **Peripheral smear (Figs 22.1A and B):**
  - **RBCs:** They show normocytic normochromic type of anemia.
  - **WBCs:** Total count is usually markedly increased. Few may present with pancytopenia. Blasts may replace normal myeloid series and can cause neutropenia.

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Figs 22.1A and B: Peripheral blood smear in acute lymphoblastic leukemia showing lymphoblasts. Inset in A shows lymphoblast with block positivity with PAS stain

- **Lymphoblasts:** The **minimum** requirement for diagnosis by WHO criteria is **20%** blasts or more. The morphologic distinctions between B and T require lymphocyte-specific markers.
- Morphology of lymphoblasts:
  - Size: Larger than small lymphocyte
  - High nucleus–to-cytoplasmic ratio
  - Nucleus: It shows condensed chromatin. Nucleoli are either absent or inconspicuous.
  - Cytoplasm: Scant to moderate agranular basophilic cytoplasm.

Lymphoblasts should be differentiated from myeloblasts (see Table 21.5).

- **Platelets:** Thrombocytopenia is common and is responsible for bleeding from skin and mucosa.

Cytochemistry of lymphoblasts (Chapter 47)

- Periodic Acid Schiff (PAS) stain demonstrates cytoplasmic aggregates of **PAS positive** (Fig. 22.1A) material **(block positivity).**
- Myeloperoxidase negative.
- Sudan Black B negative.

Bone Marrow

- **Cellularity:** Bone marrow is markedly **hypercellula**r due to proliferation of blasts which replace normal hematopoietic cells.
- **Erythropoiesis and myelopoiesis**: Reduced.
- **Megakaryopoiesis**: Megakaryocytes gradually decrease.

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Blasts

- They constitute 20–100% of the marrow cells. Lymphoblasts are described morphologically as per the French-American-British (FAB) classification as L_1, L_2 and L_3 (see Table 21.3).

Immunophenotyping

A specialized DNA polymerase called **deoxynucleotidyl-transferase (TdT),** is expressed by pre-B and pre-T lymphoblasts and is positive in ALL.

- Distinction between ALLs of precursor B and T cell origin requires lineage-specific markers.
  - Immature B cells express the pan B cell marker CD19 and CD10 (CALLA—common ALL antigen).
  - Precursor T ALL cells are positive for CD2, CD5 and CD8.

Biochemical Findings (Refer tumor lysis syndrome on page 166)

- Hyperuricemia (increased serum uric acid)
- Hyperkalemia (increased serum potassium)
- Hyperphosphatemia (increased serum phosphate)

} Due to lysis of leukemic cells

- Hypocalcemia (decreased serum calcium) due to hyperphosphatemia
- LDH is increased due to increased turnover of leukemic cells.

CSF Examination

It is required to know CNS involvement.

Prognosis

About 95% of children undergo complete remission and 75 to 85% are cured with currently available chemotherapy. Prognostic features of all are shown in Table 22.1.

Table 22.1: Prognostic factors in ALL

	Unfavorable prognosis	*Favorable prognosis*
Age	Below 2 years and above 10 years (adolescence or adulthood)	Between 2 to 10 years
Sex	Males	Females
Total WBC count	High (more than 50,000 cells/cu mm)	Low
CNS disease at presentation	Present	Absent
Cytogenetic abnormalities	t(9;22) (the Philadelphia chromosome)	Hyperploidy, trisomy of chromosomes 4, 7 and 10 and t(12;21)
Time required for clearing blasts from blood	More than 1 week	Less than 1 week

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ACUTE LYMPHOBLASTIC LYMPHOMA

About 15% of ALLs are derived from **precursor-T cell** and present in adolescent males as **lymphomas**, often with mediastinal (thymus) mass.

Lymphnode

The involved lymphnode architecture is completely effaced by lymphoblasts. Numerous mitotic figures are observed and focal "starry sky" pattern can be produced by interspersed benign tingible body macrophages that have ingested the debris of dying neoplastic cells.

SUMMARY

- Acute lymphoblastic leukemia/lymphoma (ALL) is a group of aggressive neoplasms of precursor B (pre-B) or T (pre-T) lymphocytes known as lymphoblasts.
- Proliferation of lymphoblasts in the marrow and peripheral blood are termed as lymphoblastic leukemia whereas primary involvement of lymphnodes/extranodal sites is referred to as lymphoma.
- Precursor B cell acute lymphoblastic leukemia/lymphoma is predominantly seen in children who present with symptoms due to marrow infiltration.
- Distinguishing ALL from AML only on morphological grounds may be difficult and requires cytochemistry/immunophenotyping.
- Prognosis depends on the age at the time of diagnosis, total leukocyte count and cytogenetic abnormalities.
- Precursor T cell acute lymphoblastic leukemia/lymphoma is seen predominantly in adolescent males as a mediastinal mass (thymus).

SELF-ASSESSMENT EXERCISES

I. Short Notes

1. Laboratory diagnosis of ALL
2. Peripheral smear findings in ALL
3. Prognostic factors of ALL

II. Multiple Choice Questions

1. **The following statement is TRUE in acute lymphoblastic leukemia**
   A. Testicular involvement is common
   B. It is more common in adult
   C. It may progress to mycosis fungoides
   D. Splenomegaly is less common than hepatomegaly

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2. **In ALL, the prognosis is unfavorable**
   A. In a female child
   B. When the total WBC count is low
   C. When the age of the patient is between 2 to 10 years
   D. When Philadelphia chromosome is present
3. **Both pre-B and pre-T lymphoblasts are positive for**
   A. CD5 and CD8
   B. CD19 and CD10
   C. Terminal deoxynucleotidyl-transferase
   D. Alkaline phosphatase
4. **Lymphoblasts show block positivity when stained by**
   A. PAS
   B. Myeloperoxidase
   C. Sudan black
   D. Alkaline phosphatase
5. **Following biochemical feature is NOT seen in tumor lysis syndrome**
   A. Hyperkalemia
   B. Hyperuricemia
   C. Hyperuricosuria
   D. Hypercalcemia

ANSWERS

1. A **2.** D **3.** C **4.** A **5.** D

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Chapter 23
Acute Myelogenous Leukemia and Related Neoplasm

Chapter Outline

- ❖ Acute Myelogenous Leukemia
- ❖ Myeloid Sarcoma

ACUTE MYELOGENOUS LEUKEMIA

Definition

Acute myelogenous leukemia **(AML)** is a neoplasm of hematopoietic progenitors due to acquired mutations which hinder its differentiation, resulting in **accumulation of immature myeloblasts in the marrow.**

It is also called as myeloid/myeloblastic/myelocytic leukemia.

Molecular Pathogenesis

- In AML, many recurrent genetic abnormalities disrupt genes encoding transcription factors which are involved in normal myeloid differentiation.
- **Aberrant tyrosine kinase activation** is a common feature of AML.

Thus mutated tyrosine kinase along with transcription factor aberrations result in development of AML.

Classification of acute myelogenous leukemia (see Tables 21.2 and 21.4)

Clinical Features

Age: AML may develop at any age, but is more common in adults.

Symptoms: They are related to depressed marrow function due to infiltration by neoplastic blast cells. This includes symptoms due to anemia, neutropenia and thrombocytopenia (other clinical features on page 165-166).

Laboratory Findings

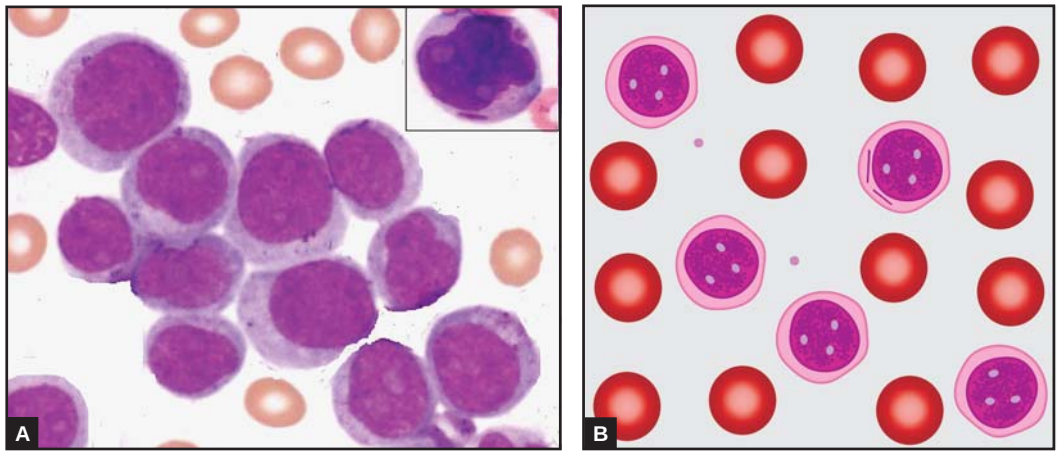
Peripheral Blood

The characteristic feature is the presence of blasts in blood and bone marrow with reduction of normal hematopoietic cells.

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- **Total WBC Count:** It is markedly raised ranging from 20×10^9/L to 100×10^9/L. In few cases count may be normal or even reduced.
- **Hemoglobin:** It is decreased and ranges from 5 to 9 gm/dL.
- **Peripheral smear (Figs 23.1A and B)**
  - **RBCs:** They show normocytic normochromic type of anemia.
  - **WBCs:** Total WBC **count** is usually **markedly increased.** Leukemic blast cells may replace normal myeloid series in the marrow and can cause neutropenia.



Figs 23.1A and B: Peripheral smear in AML with myeloblasts. Inset in: (A) Shows myeloblast with Auer rod and in: (B) One myeloblast with two Auer rods

Differential count: Shows **more than 20% myeloid blasts.** Several types of myeloid blasts are identified and leukemias may show more than one type of blast or blasts with hybrid features (see Tables 21.2 and 21.4).

Morphology of myeloblasts

Size: Myeloblasts are 3 to 5 times larger than the diameter of a small lymphocyte.

High N:C ratio

Nucleus: Fine nuclear chromatin with **2-4** variably prominent **nucleoli.**

Cytoplasm: More amount of cytoplasm than lymphoblasts. Often contains fine, azurophilic, peroxidase-positive granules.

Auer rods: They are distinctive azurophilic needle-like structures of varying length and width. They represent an abnormal alignment and crystallization of azurophilic granules and are peroxidase-positive. They are particularly numerous in AML-M3 (acute promyelocytic leukemia). These hypergranular promyelocytes with many Auer rods are known as faggot cells. **Presence of Auer rods is definitive evidence of myeloid differentiation.**

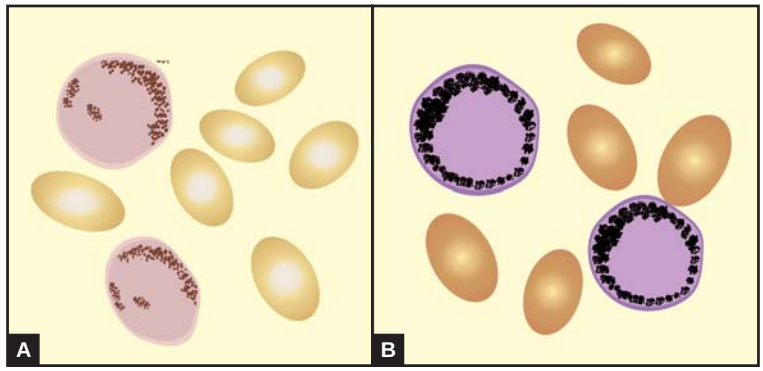
- **Platelets:** They are variably decreased. There is moderate to severe thrombocytopenia and is responsible for bleeding from skin and mucosa.

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Cytochemistry of myeloblasts (Figs 23.2A and B)

Myeloblasts **stain positively** with **myeloperoxidase (MPO) and Sudan black B.** Monoblasts stain with nonspecific esterases (refer Chapter 47).



Figs 23.2A and B: Myeloblast stained positively with myeloperoxidase: (A) and Sudan black (B)

Bone Marrow

- **Cellularity:** Bone marrow is markedly **hypercellular** due to proliferation of blasts which replace normal hematopoietic cells.
- **Erythropoiesis:** Markedly suppressed.
- **Myelopoiesis:** It shows suppression of myeloid maturation and **myeloblasts constitute more than 20% of marrow cells.**
- **Megakaryopoiesis:** Megakaryocytes are **gradually decreased**. Megakaryocytes usually show dysmegakaryopoiesis in the form of monolobate and hyperlobate forms.

Biochemical Findings (refer tumor lysis syndrome on page 166)

- **Hyperuricemia** (increased serum uric acid)
- **Hyperkalemia** (increased serum potassium)
- **Hyperphosphatemia** (increased serum phosphate)

} Due to lysis of leukemic cells

- **Hypocalcemia** (decreased serum calcium) due to hyperphosphatemia.
- **Serum lysozyme** increased especially in monocytic subtypes (AML M4 and M5).

Positive FDP and Decreased Fibrinogen

In AML-M3, promyelocytes release tissue factor like substance which leads to DIC, which shows the presence of FDP and reduced levels of fibrinogen in the plasma.

Immunophenotyping

Since distinction between myeloblasts and lymphoblasts on morphological features alone is difficult, the diagnosis of AML is confirmed by using stains for myeloid specific antigens (see Table 21.6).

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Cytogenetics

- Cytogenetic analysis is very important in the WHO classification of AML (see Table 21.4).
- Particular chromosomal abnormalities correlate with certain clinical features.
- Balanced chromosomal translocations, particularly t(8;21), inv(16) and t(15;17) are associated with AMLs arising de novo in young adults.
- AMLs which follow MDS or exposure to DNA-damaging agents (like chemotherapy or radiation therapy) commonly have chromosomal abnormalities like deletions or monosomies involving chromosomes 5 and 7 and are without chromosomal translocations.
- AML in elderly is associated with deletions of chromosomes 5q and 7q.

Prognosis

It has a fulminant course and has worse prognosis than ALL. Cytogenetic markers are major determinants of prognosis. The prognosis in each sub categories is presented in see Table 21.4.

MYELOID SARCOMA

Definition: It is defined as a tumor mass consisting of myeloid blasts with or without maturation occurring at an anatomical site other than bone marrow (extramedullary sites).

It is also known as **extramedullary myeloid tumor/granulocytic sarcoma/chloroma.** Infiltrate of myeloid blasts in leukemic patients developing at anywhere in the body, unless they present with tumor masses (with effaced tissue architecture), are not classified as myeloid sarcoma.

Etiology: It is same as that for acute myeloid leukemia (AML) and myeloproliferative neoplasm (MPN).

Sites of involvement: Any site may be involved. More frequently found in skin, lymphnode, GI tract, bone, soft tissue and testis.

Morphology:

- **On sectioning** the fresh tumor mass, it appears **green (**hence the term **chloroma)**, probably due to the enzyme myeloperoxidase. The green color changes to dirty yellow on exposure to atmospheric air.
- **Microscopically** it consists of **myeloblasts** with or without features of promyelocytic or neutrophilic maturation that partially or completely effaces the tissue architecture.

SUMMARY

- AML can develop at any age and is more common in adults.
- WHO classification of acute leukemia depends on the morphology, cytochemistry, cytogenetics, molecular genetics and clinical features. According to the revised WHO (2008) classification, AML is divided into seven major categories.
- The clinical presentation is similar to ALL and is mainly due to myeloid blasts infiltrating the bone marrow elements.

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- Peripheral smear shows myeloblasts, the appearance of which depends on the subtype. Auer rods are a definite evidence of myeloid differentiation.
- Differentiation of myeloblast from lymphoblast may be aided by cytochemical stains and/or by immunophenotyping.

SELF-ASSESSMENT EXERCISES

I. Essays

1. Define and classify leukemia. Describe the laboratory diagnosis of acute myeloid leukemia.
2. Classify AML and describe its peripheral smear and bone marrow findings.

II. Short Notes

1. Peripheral blood smear and bone marrow findings in acute myelogenous leukemia
2. Faggot cell
3. Auer rods

III. Multiple Choice Questions

1. **The morphological type of leukemia which may show Auer rods is**
   A. Acute myeloid leukemia
   B. Chronic myeloid leukemia
   C. Chronic lymphoid leukemia
   D. Acute lymphoblastic leukemia
2. **Auer rods are found in the cytoplasm of**
   A. Lymphoblast
   B. Myeloblast
   C. Monoblasts
   D. Megaloblast
3. **Auer rods represent**
   A. Ribosomes
   B. Mitochondria
   C. Azurophilic granules
   D. Secondary granules
4. **Hypergranular promyelocytes with faggot cells are found in AML of FAB type**
   A. M1
   B. M2
   C. M3
   D. M4

ANSWERS

1. A **2.** B **3.** C **4.** C

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CHAPTER 24 Myelodysplastic Syndromes

CHAPTER OUTLINE

- Definition
- Classification
- Etiology and Molecular Pathogenesis
- Clinical Features
- Laboratory Findings

DEFINITION

Myelodysplastic syndromes (MDS) are a heterogeneous group of acquired clonal stem cell disorders characterized by progressive cytopenias, dysplasia in one or more cell lines, ineffective hematopoiesis and risk of development of AML.

CLASSIFICATION

- ***Idiopathic or primary MDS*:** It is a disease of **elderly** usually over 50 years of age and often develops insidiously.
- ***Secondary/therapy-related MDS (t-MDS):*** It is a complication of previous cytotoxic drug or radiation therapy. Certain inherited hematological disorders like Fanconi anemia, dyskeratosis congenita and Diamond-Blackfan syndrome are also associated with increased risk of MDS.

WHO Classification of Myelodysplastic Syndromes (Table 24.1)

The morphological classification of MDS is based on: (1) the presence of blasts in the bone marrow and peripheral blood, (2) the type and degree of dysplasia and (3) the presence of ring sideroblasts.

Table 24.1: WHO classification, peripheral smear and bone marrow findings of myelodysplastic syndromes

Disease	*Peripheral smear*	*Bone marrow*
Refractory cytopenia with unilineage dysplasia (RCUD): Refractory anemia (RA), refractory neutropenia (RN), refractory thrombocytopenia (RT)	Unicytopenia or bicytopenia[1]	Unilineage dysplasia in ≥10% of the cells in one myeloid lineage <5% blasts <15% ring sideroblasts
Refractory anemia with ring sideroblasts (RARS)	Anemia No blasts	Dyserythropoiesis ≥15% ring sideroblasts <5% blasts

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Refractory cytopenia with multilineage dysplasia (RCMD)	Bi/pancytopenia Rare blast No Auer rods <1 × 10^9/L monocytes	Dysplasia in ≥10% of cells in 2 myeloid lineages (neutrophil and/or erythroid and/or megakaryocytes) <5% blasts No Auer rods ± 15% ring sideroblasts
Refractory anemia with excess blasts-1(RAEB-1)	< 5% blasts[2] Bi/pancytopenia No Auer rods <1 × 10^9/L monocytes	5-9% blasts Unilineage or multilineage dysplasia No Auer rods
Refractory anemia with excess blasts-2 (RAEB-2)	5-19% blasts Cytopenia Auer rods ±[3] <1 × 10^9/L monocytes	10-19% blasts Unilineage or multilineage dysplasia Auer rods ±[3]
MDS unclassified (MDS-U)	≤1% blasts Cytopenia only	<5% blasts Unequivocal dysplasia in less than 10% of cells in one or more myeloid cell lines when accompanied by cytogenetic abnormalities considered as presumptive evidence for diagnosis of MDS
MDS with isolated del (5q)	No or rare blasts Anemia Platelets increased or normal	<5% blasts Increased to normal megakaryocytes with hypolobated nuclei. Isolated 5q deletion No Auer rods

1. Cases with pancytopenia should be classified as MDS-U
2. If marrow blasts <5% with 2-4% myeloblasts in blood = RAEB-1. Cases with RCUD and RCMD with 1% myeloblasts in blood should be classified as MDS-U
3. Cases with Auer rods and <5% myeloblasts in blood and 10% in marrow = RAEB-2

ETIOLOGY AND MOLECULAR PATHOGENESIS

In primary MDS the exact cause is not known, however genetic factors may play a role. The causes of secondary /t-MDS are radiation, benzene, post-chemotherapy, viruses, cigarette smoking and PNH. Even though MDS is a group of heterogeneous diseases, a common factor is that they are the result of proliferation of abnormal stem cells. The exact pathogenesis is not known.

- The initiating defect in most cases is at the level of common myeloid precursor cell, because MDS rarely transforms into acute lymphoid leukemia.
- The causative factors lead to stem cell mutation and proliferation.
- There is also increased apoptosis of bone marrow progenitors causing ineffective hematopoiesis. The consequences are progressive cytopenias with dysplasia in one or more cell lines (erythroid, myeloid or megakaryocytic).
- The common chromosomal abnormalities noted are monosomies 5 and 7, deletions of 5q, 7q and 20q and trisomy 8.

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CLINICAL FEATURES

Usually found in elderly patients above 60 years and slightly more common in males. They are detected incidentally on routine blood examination in about 50% of patients. Symptoms are due to cytopenias which may be single-lineage cytopenia, bicytopenia or pancytopenia. Symptoms include weakness (anemia), infections (leukopenia) and hemorrhage (thrombocytopenia). In about 10 to 40% of patients, MDS progresses to AML because of which MDS was previously referred to as preleukemic syndrome.

LABORATORY FINDINGS

Peripheral Smear

The abnormal hematopoietic cells undergo increased apoptosis in the bone marrow resulting in cytopenias in the peripheral blood.

- **RBCs:** They show mild to moderate degree of macrocytic or dimorphic anemia with evidence of dyspoiesis.
- **WBCs:**
  - **Count:**
    - Normal or low total leukocyte count.
    - Neutropenia with blasts. Variable number of blasts determines the type of MDS (see Table 24.1).
  - **Cell:** Large and the cytoplasm is hypogranular or without granules (agranular).
  - **Nuclei:** Hyposegmentation of neutrophils with only two nuclear lobes (pseudo-Pelger-Hüet cells), hypersegmenation or ringed neutrophils.
- **Platelets:** Variable thrombocytopenia, presence of large hypogranular or giant platelets is seen.
- **NAP score** is moderately or severely decreased.

Bone Marrow

The most characteristic feature is the varying degree of dysplasia/dyspoietic (disordered) differentiation (Table 24.2) affecting all non-lymphoid lineages (erythroid, granulocytic, monocytic and megakaryocytic) associated with cytopenias.

- **Cellularity**: Usually hypercellular due to partial or complete replacement by clonal mutant multipotent stem cells, but may be normocellular or rarely hypocellular (hypocellular MDS). Evidence of dysplasia in one or more hematopoietic cells depends on the lineage involved.
- **Erythropoiesis:** Dysplastic changes are more frequent in erythroid precursors and they show dyserythropoiesis. Dysplastic changes in erythroid precursors are:
  - **Nuclear changes:** The nuclei may show multinucleation, **megaloblastoid** change, and abnormal nuclear shapes**.** Instead of the normal round shape of nuclei, they may show lobes, budding (nuclei with misshapen, often polyploidy outlines), bridging between nuclei (internuclear bridging) and karyorrhexis (fragmentation).

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  - **Cytoplasmic changes: Ringed sideroblasts** (erythroid precursors with perinuclear arrangement of mitochondrial iron and show positive Prussian blue reaction-refer see Fig. 17.1), vacuolization and periodic acid-Schiff positivity.
- **Myelopoiesis:** It shows **hyperplasia with dysgranulopoiesis** which is characterized by nuclear hyposegmentation (pseudo-Pelger—Hüet cells) hypersegmentation and cytoplasmic hypogranulation. Monocytic hyperplasia is also commonly found. Myeloblasts may be increased but make up less than 20% of the overall marrow cellularity.
- **Megakaryopoiesis:** It shows **dysmegakaryopoiesis.** Megakaryocytic dysplasia is characterized by single nuclear lobes (mononuclear or hypolobated forms) or multiple separate nuclei (**pawn ball megakaryocytes)** or micromegakaryocytes.
- **Iron stores:** Because of ineffective erythropoiesis iron stores are **increased**.

Ineffective hematopoiesis: Mutant stem cells have capacity to differentiate into red cells, granulocytes and platelets but in an ineffective and disordered fashion.

Bone marrow trephine biopsy: It reveals abnormal localization of immature precursors (ALIP) in RAEB. The clusters of blast cells are present in central parts of the marrow. Megakaryocytic dysplasia is highlighted better in trephine biopsy.

Morphological Features of Dysplasia (Table 24.2)

Table 24.2: Morphological features of dysplasia in peripheral smear and bone marrow in MDS

	Peripheral smear	*Bone marrow*
Dyserythropoiesis	Oval macrocytes Hypochromic microcytes Dimorphic RBC picture	**Nuclear features:** Nuclear budding, intranuclear bridging, karyorrhexis, multinucleation, hyperlobation and megaloblastoid changes. **Cytoplasmic features:** Ring sideroblasts, vacuolization Periodic acid-Schiff positivity.
Dysgranulopoiesis	Persistent basophilic cytoplasm in mature WBC Abnormal granulation Abnormal nuclear shapes	**Size:** Small or unusually large **Nucleus:** Nuclear hyposegmentation (pseudo-Pelger-Hüet; pelgeroid) irregular hypersegmentation. **Cytoplasm:** Hypogranulation, Auer rods
Dysmegakaryopoiesis	Giant platelets Platelets with abnormal granulation (hypo or agranular), Circulating micromegakaryocytes	Nuclear hypolobation, Megakaryocytes with multiple separate nuclei **(pawn ball megakaryocytes)**, micromegakaryocytes

SUMMARY

- MDS are a group of heterogeneous acquired clonal defect at the stem cell level with dysplasia in one or more cell lines (myeloid, erythroid, megakaryocytic).
- They produce several structural abnormalities during maturation and premature cell destruction.

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- MDS are characterized by peripheral cytopenia, hypercellular bone marrow and dysplasia in the affected cell lineage.
- Progression to acute leukemia is seen in significant number of cases.

SELF-ASSESSMENT EXERCISES

I. Short Note

Myelodysplastic syndromes

II. Multiple Choice Questions

1. **The type of anemia usually seen in MDS is**
   A. Microcytic, hypochromic
   B. Normocytic normochromic
   C. Normocytic hypochromic
   D. Macrocytic normochromic
2. **Myelodysplastic syndrome has following characteristics EXCEPT**
   A. It is a congenital stem cell disorder
   B. It is characterized by dysplasia in non-lymphoid lineage
   C. It shows megaloblastoid and ineffective hematopoiesis
   D. There is a risk of development of AML
3. **The following disorder shows pawn ball megakaryocytes**
   A. Pernicious anemia
   B. Hodgkin lymphoma
   C. Myelodysplastic syndrome
   D. Immune thrombocytopenic purpura

ANSWERS

1. D **2.** A **3.** C

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CHAPTER 25 Myeloproliferative Neoplasm

CHAPTER OUTLINE

- Introduction
- Essential Thrombocythemia
- Primary Myelofibrosis

INTRODUCTION

Definition

Myeloproliferative neoplasms are clonal hematopoietic stem cell disorders which are characterized by **proliferation of one or more of the myeloid lineages** (erythroid, granulocytic, megakaryocytic and mast cells).

It is seen usually in adults with a peak in the 5th to 7th decade. Splenomegaly and hepatomegaly occurs commonly due to sequestration of excess hematopoietic cells or proliferation of abnormal hematopoietic cells.

WHO Classification of Myeloproliferative Neoplasm (MPN): It is presented in Table 25.1

Table 25.1: Comparison of WHO 2001 and 2008 classification of myeloproliferative neoplasm (MPN)

WHO 2001 Chronic myeloproliferative disorders	*WHO 2008 Myeloproliferative neoplasms*
Chronic myeloid leukemia	Chronic myelogenous leukemia, BCR-ABL-1 positive
Chronic neutrophilic leukemia	Chronic neutrophilic leukemia
Polycythemia vera	Polycythemia vera – JAK2 V617F or exon 12 mutation
Chronic idiopathic myelofibrosis	Primary myelofibrosis – JAK2 or MPL mutation
Essential thrombocythemia	Essential thrombocythemia
- platelet count > 600 × 10^9 /L	- Platelet count > 450 × 10^9 /L
	- JAK2 mutation
Chronic eosinophilic leukemia	Chronic eosinophilic leukemia, NOS
	- No BCR-ABL1, PDGFRA, PDGFRB or FGFR1 translocation
Mastocytosis was not included in this category earlier	Mastocytosis – KIT mutation
Chronic myeloproliferative disease, unclassifiable	Myeloproliferative neoplasm, unclassifiable

- Most myeloproliferative neoplasms originate in multipotent myeloid progenitors, whereas others arise in pluripotent stem cells (which give rise to both lymphoid and myeloid cells).

Pathogenesis

- The common pathogenic feature is the presence of mutated, constitutively activated tyrosine kinases.
- **Normally,** the hematopoietic growth factors act by binding to surface receptors present on the marrow progenitor cells and activate tyrosine kinases.

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- The mutated constitutively activated tyrosine kinases result in the growth factor–independent proliferation and survival of marrow progenitors. Proliferation of hematopoietic stem cells leads to a hypercellular marrow. The proliferating cells undergo normal differentiation, which results in increased blood counts of one or more mature blood elements like mature granulocytes, erythrocytes or platelets.

Note: Chronic myelogenous leukemia (see Chapter 26) and polycythemia vera (see Chapter 18) are discussed separately.

ESSENTIAL THROMBOCYTHEMIA

Definition

Essential thrombocythemia (ET) is a chronic myeloproliferative neoplasm (MPN) which involves primarily megakaryocytic lineage. It is characterized by increased megakaryopoiesis and thrombocytosis (more than 450 × 10^9/L). This is also known as **primary (essential/idiopathic) thrombocytosis**.

Etiology

- Exact etiology is unknown. It is associated in majority of the cases with point mutations in JAK2 (50%) or MPL (5–10%), a receptor tyrosine kinase which is normally activated by thrombopoietin.
- When there is no mutation in the tyrosine kinase, secondary causes of reactive thrombocytosis (like chronic inflammation/infection, chronic blood loss and iron deficiency) must be ruled out before the diagnosis of ET.
- Constitutive activation of JAK2 or MPL signaling leads to thrombopoietin-independent proliferation of megakaryocytes. The JAK2 mutation is similar to that observed in PV (page 133-134).

Clinical Features

It usually occurs at the age of 50-60 years. Common and important clinical features are due to *platelet dysfunction causing thrombosis and hemorrhage.* They may present with deep venous thrombosis, portal and hepatic vein thrombosis and myocardial infarction. Throbbing and burning sensation of hands and feet due to blocking of small arterioles by platelet aggregates, referred to as *erythromelalgia, is one of the characteristic feature.*

Laboratory Findings

Peripheral Smear

- **RBCs:** Show normocytic normochromic picture.
- **WBCs:** Show mild leukocytosis.
- **Platelets:** Increased number of platelets with variation in size and shape are seen. Abnormally large platelets are common. Platelets may form clusters and accumulate near the thin edge of the smear.

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Bone Marrow

- **Cellularity:** Mild to marked hypercellularity is seen
- **Erythropoiesis:** Normal or shows mild hyperplasia
- **Myelopoiesis:** Normal or shows mild hyperplasia.
- **Megakaryopoiesis:** They are markedly increased in number, **abnormally large (giant megakaryocytes)** and with features of dysmegakaryopoiesis (see Table 24.2).

Bone Marrow Biopsy

It shows reticulin fibrils with megakaryocyte clustering.

Extramedullary Hematopoiesis

It may result in mild hepatosplenomegaly. Rarely, a spent phase of marrow fibrosis or transformation to AML may develop.

Platelet Function

Platelet may show qualitative abnormalities. Aggregation studies demonstrate lack of response to epinephrine.

Course

ET is an indolent disorder with asymptomatic periods interrupted by occasional thrombotic episodes or hemorrhage.

PRIMARY MYELOFIBROSIS

Definition

Primary myelofibrosis is a clonal MPN characterized by a proliferation of predominantly megakaryocytes and granulocytes in the bone marrow. In the fully developed disease, the reactive marrow fibrosis replaces hematopoietic cells leading to cytopenias and extensive extramedullary hematopoiesis.

Molecular Pathogenesis

Most cases show activating JAK2 mutations and activating MPL mutations.

- The bone marrow shows extensive deposition of collagen by non-neoplastic fibroblasts. The fibrosis replaces the hematopoietic elements, including stem cells, leading to marrow failure.
- It may be due to inappropriate release of fibrogenic factors (platelet-derived growth factor and TGF-β) from neoplastic megakaryocytes in the marrow.
- As marrow fibrosis progresses, circulating hematopoietic stem cells reside in secondary hematopoietic organs (spleen, liver and lymph nodes) causing extramedullary hematopoiesis.
- Even though the blood cells are produced at extramedullary sites, they are disordered and this factor along with bone marrow failure results in moderate to severe cytopenias.

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Clinical Features

Primary myelofibrosis is usually found above 60 years of age and is less common than PV and ET. The symptoms are due to progressive anemia and splenomegaly (sensation of fullness in the left upper quadrant). Increase in metabolism and expanding mass of hematopoietic cells may cause symptoms like fatigue, weight loss and night sweats. Other manifestations include hyperuricemia and secondary gout due to a high rate of cell turnover.

Laboratory Findings

Peripheral Smears

- **RBCs:** They show moderate to severe degree of normochromic normocytic anemia accompanied by leukoerythroblastosis. **Teardrop-shaped red cells** (dacryocytes), probably due to damage in the fibrotic marrow can also be found.
- **WBCs:** Total white cell count is usually normal or reduced, but can be markedly elevated 80 to 100 × 10^9/L in early stages of the disease.
- **Platelets:** They may be abnormally large. The platelet count is usually normal or elevated, but as the disease progresses the count decreases.

Bone Marrow

- **Cellularity:** In early stages, it is often hypercellular due to increase in maturing cells of all lineages. In later stags, it is replaced by fibrosis and becomes hypocellular and diffusely fibrotic resulting in a **dry tap.**
- **Erythroid and granulocytic precursors** are morphologically **normal.**
- **Megakaryocytes are large, dysplastic and abnormally clustered**.

Bone Marrow Biopsy

- It is essential for the diagnosis of myelofibrosis as aspirate results in a dry tap late in the course of the disease.

Stages: **Two stages** have been recognized.

1. **Prefibrotic (cellular) stage:** This stage is characterized by a hypercellular bone marrow. Megakaryocytes are increased in number and markedly abnormal. They form abnormal clusters of variable sizes adjacent to vascular sinusoids and the bony trabeculae. The megakaryocytes have abnormal nuclear cytoplasmic ratio, abnormal chromatin clumping and nucleus is bulbous, cloud like or balloon shaped.
2. **Fibrotic stage:** The marrow fibrosis results in characteristic peripheral blood findings. Fibrosis distorts the marrow and prematurely releases nucleated erythroid and early granulocyte progenitors **(leukoerythroblastosis)** from the marrow and from extramedullary hematopoietic sites. In later stages, the hematopoietic elements are found within dilated sinusoids, which indicate severe architectural distortion caused by fibrosis. In severe cases, fibrotic marrow spaces may be transformed into bone ("osteosclerosis"). Reticulin stain is mandatory to demonstrate the increase in reticulin fibers (fibrosis).

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Extramedullary Hematopoiesis

- Marrow fibrosis causes extensive extramedullary hematopoiesis in organs like **spleen and liver.**
- **Spleen** is usually markedly enlarged (even up to 4000 gm). Grossly, spleen is firm and red to gray in color. **Liver** may be moderately enlarged. Hematopoiesis can also develop in lymph nodes.

Course: The course is variable and the median survival ranges from 3 to 5 years. In 5 to 20% of cases, it transforms to AML. It may be complicated by intercurrent infections, thrombotic episodes and bleeding due to platelet abnormalities.

SUMMARY

- Myeloproliferative neoplasms are clonal hematopoietic stem cell disorders characterized by proliferation of one or more of the myeloid lineages. The disorders mainly include chronic myelogenous leukemia, polycythemia vera, essential thrombocythemia and primary myelofibrosis.
- Essential thrombocytosis is a clonal MPN with increased megakaryopoiesis and thrombocytosis. ET is observed above 60 years of age and clinical features are due to platelet dysfunction causing thrombosis and hemorrhage.
- Primary myelofibrosis is an MPN characterized by marrow fibrosis with cytopenias and extensive extramedullary hematopoiesis. Two stages have been recognized namely, prefibrotic and fibrotic stage.

SELF-ASSESSMENT EXERCISES

I. Short Note

Myeloproliferative neoplasms

II. Multiple Choice Questions

1. **The following is NOT a feature of essential thrombocythemia**
   A. Thrombocytosis more than 450 × 10^9/L.
   B. Majority associated with point mutations in JAK2
   C. Usually observed below the age of 20 years
   D. Show erythromelalgia
2. **Confirmation of myelofibrosis is usually done by**
   A. Bone marrow aspiration
   B. Bone marrow biopsy
   C. Splenic aspiration
   D. Immunophenotyping

ANSWERS

1. C **2.** B

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Chapter 26 Chronic Myelogenous Leukemia and Other Myeloid Neoplasms

Chapter Outline

- ❖ Chronic Myelogenous Leukemia
- ❖ Myelodysplastic/Myeloproliferative Neoplasms

CHRONIC MYELOGENOUS LEUKEMIA

Definition

Chronic myelogenous (myelocytic/myeloid/granulocytic) leukemia (CML) is an **acquired clonal myeloproliferative neoplasm** of the abnormal pluripotent **hematopoietic stem cell.**

It is distinguished from other myeloproliferative neoplasms by the presence of chimeric fusion BCR-ABL gene and characteristic chromosomal abnormality, i.e. Philadelphia (Ph) chromosome in more than 90% of cases. It is characterized by neoplastic proliferation causing excessive production and reduced apoptosis of cells of the myeloid series in the bone marrow.

Etiology and Pathogenesis

In majority of cases the **etiology** is **not known.** Exposure to ionizing radiation may increase the risk and is dose-dependent. The evidences which support this risk factor are:

- Increased incidence of CML in survivors of Hiroshima and Nagasaki atomic blasts.
- Patients who have received radiation for cancer therapy have higher chances of developing CML compared to normal individuals.

Molecular Pathogenesis (Figs 26.1 and 26.2)

Philadelphia chromosome:

- Philadelphia (Ph) chromosome is an acquired chromosomal abnormality **present in all proliferating hematopoietic stem cells** (erythroid, myeloid, monocytic and megakaryocytic precursors). Rarely, it is found in B and T lymphocytes, but never in marrow fibroblasts.
- **Philadelphia chromosome** is a **balanced reciprocal translocation** between long arm of chromosome 9 and chromosome 22, i.e. **t (9; 22) (q 34; q 11.2)**. This results in an increase in the length of chromosome 9 and a shortened long arm of one of the chromosome 22. This **shortened chromosome 22** is known as Philadelphia chromosome (Fig. 26.1).

BCR-ABL fusion gene:

- At the molecular and genetic level, the proto-oncogene namely ***ABL*** (Abelson murine leukemia virus) gene from chromosome 9 **joins** the ***BCR*** (breakpoint cluster region) on

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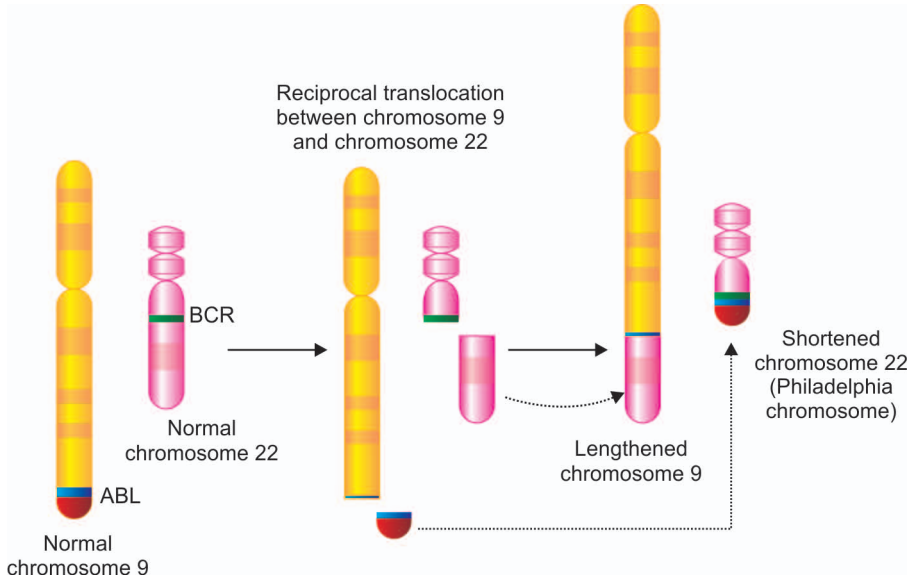


Fig. 26.1: Balanced reciprocal translocation between long arm of chromosome 9 and chromosome 22 resulting in shortened chromosome 22 known as Philadelphia chromosome

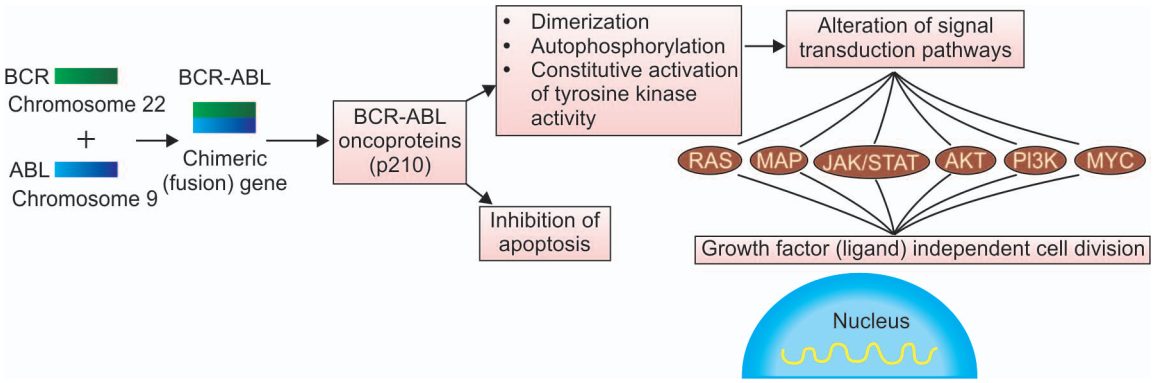


Fig. 26.2: Fusion of ABL gene from chromosome 9 with BCR on chromosome 22 and its consequences

chromosome 22. This result in a new **chimeric (fusion) gene called BCR-ABL** which is hallmark of CML and critical for the molecular events (Fig. 26.2).

- **Normally,** ABL is a proto-oncogene which regulates tyrosine kinase activity. Tyrosine kinases serve as growth factor receptors and normally regulate ligand-mediated dimerization and autophosphorylation.
- **In CML, BCR-ABL** fusion results in **conversion of ABL proto-oncogene into** a functioning **oncogene** with enhanced tyrosine kinase activity.
- The most **important oncoprotein** produced by BCR-ABL oncogene is **p210.** The oncoprotein binds directly to the adaptor proteins which induces signal transduction pathway without

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activation of growth factor receptors. [**Normally**, signals are generated with ligand binding of growth factor receptors. The signal transduction pathways involved are RAS, mitogen-activated-protein (MAP) kinases, JAK/STAT, AKT, phosphatidyl inositol 3-kinase (PI3K), MYC and others].

- The net effect of these events is **cell division and inhibition of apoptosis** independent of ligand binding. The proliferating CML cells compete with normal cells and suppress the normal hematopoiesis.
- BCR-ABL not only causes proliferation of granulocytic (megakaryocytic to a lesser degree) progenitors, but also the abnormal release of immature granulocytic forms into the peripheral blood from the marrow.

Depending on the presence or absence of Philadelphia chromosome and BCL-ABR, the chronic myeloid leukemia can be grouped into three categories (Table 26.1).

Table 26.1: Grouping of CML according to Philadelphia chromosome and BCL-ABR

Ph	*BCR-ABL*	*Survival rate and therapeutic response*
Positive	Positive	Better in both types
Negative	Positive	
Negative	Negative	Worse prognosis

Clinical Features

CML is the **commonest leukemia** and constitutes about 25-30% of all leukemias in India.

***Age*:** It is primarily seen in the **fifth and sixth decades** of life.

Sex: Males are affected slightly more than females.

Onset: It is gradual in onset with vague **nonspecific symptoms**. About 50% of patients remain asymptomatic and are diagnosed during routine medical or blood examination.

- **Anemia** and hyper metabolism due to rapid turnover of cells may result in symptoms like fatigue, weakness, weight loss, anorexia and often patient complains of **fullness of abdomen due to splenomegaly.**
- **Rare presentations**:
  - **Bleeding** (associated with a low platelet count and/or platelet dysfunction).
    - Retinal hemorrhages may cause visual disturbances.
    - Raised histamine levels due to basophilia may result in upper gastrointestinal ulceration and bleeding.
  - **Venous thrombosis** (due to thrombocytosis and/or marked leukocytosis/ leukostasis).
  - **Leukostatic symptoms**:
    - Dyspnea, drowsiness, loss of coordination or confusion (due to sludging in the pulmonary or cerebral vessels). These symptoms are frequent only in the accelerated or blastic phases.
    - **Priapism:** Painful penile erection due to leukostasis (associated with marked leukocytosis or thrombocytosis).
  - **Gouty arthritis:** Due to elevated uric acid levels resulting from high turnover of leukemic cells.

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Physical Examination

- **Pallor:** It is due to anemia.
- **Sternal tenderness and bone pain:** This is due to hypercellularity of marrow and irritation of periosteum.
- **Splenomegaly:** It is characteristically observed and is due to leukemic infiltration and extramedullary hematopoiesis.
- **Lymphadenopathy:** If present indicates progression to accelerated or blast phase of CML.

Natural History of Chronic Myeloid Leukemia

CML is a slowly progressing disease with a survival rate of 3 to 5 years even without treatment. There are **three different phases.**

Chronic/Stable/Indolent Phase (CP)

- Most of the patients are diagnosed in the chronic phase
- It usually lasts for 2 to 6 years
- If not treated properly, it gradually transforms in few months to accelerated phase or suddenly to blastic phase (crisis), which is fatal.

Laboratory Findingsc

Peripheral blood

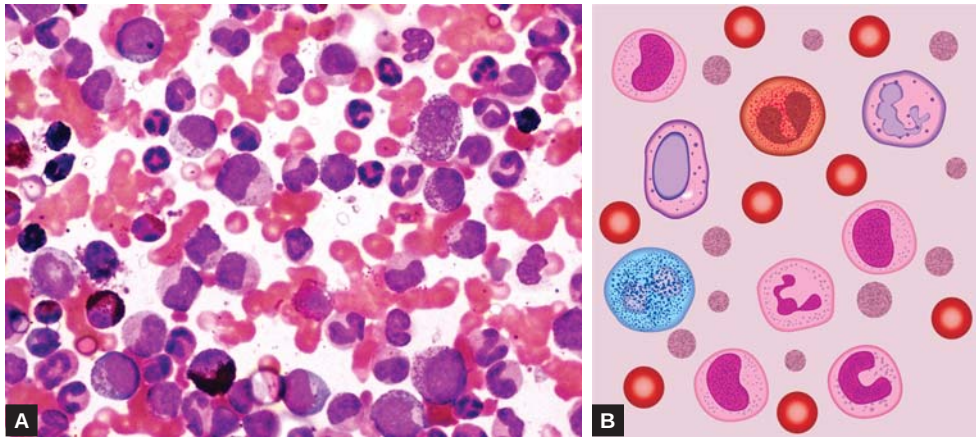
- **Hemoglobin:** It is usually less than 11 gm/dL.
- **Peripheral smear**
  - **RBCs:** Show moderate degree of **normocytic normochromic anemia.** Severity of anemia is proportional to the increase in leukocytes. The red cells may show mild variation in size
  - **WBCs**
    - These are characterized by marked **leukocytosis** (12-600 × 10^9/L). The **total leukocyte count** usually **exceeds 100 × 10^9/L (1,00,000/cu mm)**. In untreated patients, the leukocyte count progressively increases.
    - **Shift to left (shift to immaturity)** with granulocytes at all stages of development (neutrophils, metamyelocytes, myelocytes, promyelocytes and an occasional myeloblasts) are found in the peripheral blood (Table 26.2). **Predominant cells** are **neutrophils and myelocytes** in an untreated patient. **Blasts** are usually **less than 10%** of the circulating white blood cells (Figs 26.3A and B).
    - **Basophils and eosinophils:** Basophilia and eosinophilia are usually observed.
    - **Decreased NAP/LAP score:** NAP score in CML is decreased and is usually below 20 (normal score range is 40-100) in majority of patients. This is helpful in differentiating CML from leukemoid reaction (see Table 19.4).
- **Platelets:** Platelets range from normal (150-450 × 10^9/L) to greater than 1000 × 10^9/L. Up to 50% of patients have **thrombocytosis** (increased platelets). Thrombocytopenia in CML is an indication of an impending accelerated or blast phase.

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Table 26.2: Differential leukocyte count in a classical untreated CML

Cell	*Percentage*
Blasts	<10
Promyelocytes	4-8
Myelocytes	**20-30**
Metamyelocytes	15-25
Band forms	5-15
Neutrophils	**40-70**
Basophils	1-8
Eosinophils	2-10



Figs 26.3A and B: Peripheral blood picture in chronic stable phase of chronic myeloid leukemia

Bone marrow

- **Cellularity:** Markedly **hypercellular** due to marked **myeloid hyperplasia**.
- **M:E ratio:** Often exceeds **20:1**.
- **Erythropoiesis:** Myeloid hyperplasia replaces the marrow erythroid cells resulting in **diminished erythropoiesis** and anemia.
- **Myelopoiesis:** Shows **marked hyperplasia.** Blast cells are usually less than 10%. Basophils, eosinophils and their precursors are usually found.
- **Megakaryopoiesis:** Megakaryocytes are either normal or increased. There are usually small, dysplastic forms of megakaryocytes **(dwarf megakaryocytes)** along with megakaryocytic clustering.
- **Sea-blue histiocytes (Gaucher-like cells/pseudo Gaucher cells):**
  - Bone marrow often shows the presence of scattered large storage histiocytes with wrinkled, green-blue cytoplasm (acquired sea-blue histiocytes).
  - These cells reflect increased intramedullary cell turnover. The histiocytes have increased cerebrosides which cannot be processed by the normal or increased amount of β-glucocerebrosidase.
  - The histiocytes in Gaucher disease lack the enzyme β-glucocerebrosidase.
- **Fibrosis of the bone marrow:** Reticulin stain reveals increased deposition of reticulin fibers.

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Philadelphia chromosome and ***BCR-ABL fusion gene*** can be demonstrated either by chromosomal analysis or **fluorescent *in situ* hybridization (Fig. 26.4)** or PCR based tests.

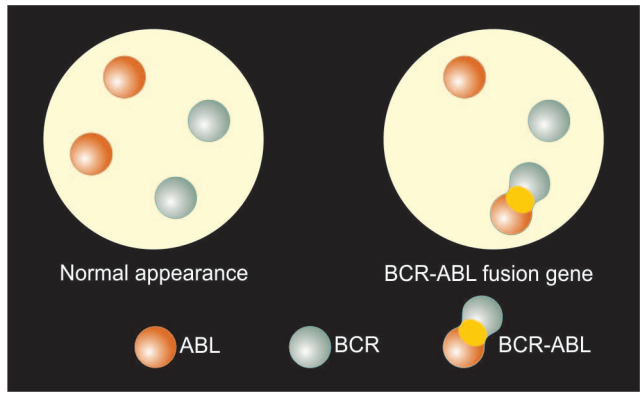


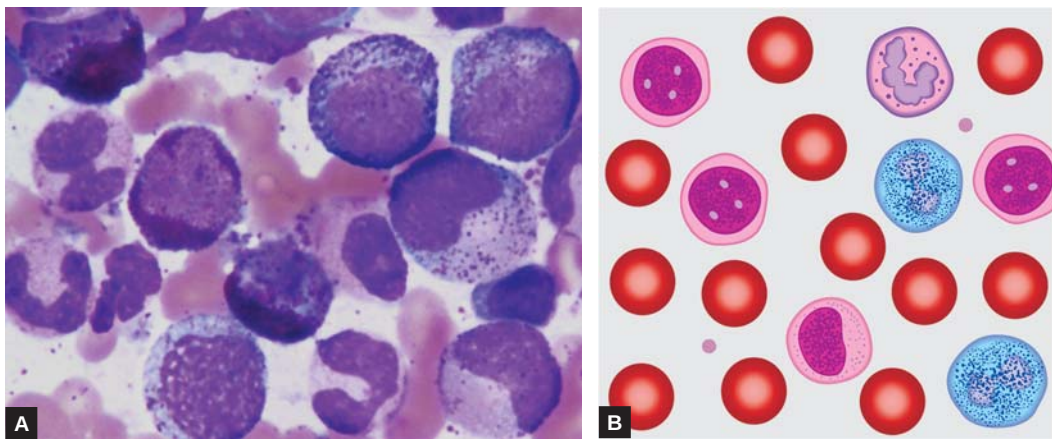
Fig. 26.4: Fluorescent *in situ* hybridization showing BCR-ABL fusion gene

Biochemical findings

- Serum uric acid is increased due to high turnover of leukemic white cells.
- Serum LDH is increased.
- Serum alkaline phosphatase is increased.
- Serum vitamin B_{12} is elevated due to production of binding protein by the granulocyte series.

Accelerated Phase (AP)

- It is a more aggressive phase which lasts for a few months.
- **Peripheral blood and bone marrow (Figs 26.5A and B):** It is characterized by
  - Increasing anemia.
  - Persistent or increasing WBC count (more than 10×10^9/L) unresponsive to therapy.



Figs 26.5A and B: Peripheral blood picture in accelerated phase of chronic myeloid leukemia showing numerous blasts (10-19%) and striking basophilia

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- Myeloblasts 10-19% are seen in the blood or bone marrow.
- Striking basophilia (20% or more).
- Persistent thrombocytopenia (less than 100×10^9/L) unrelated to therapy or persistent thrombocytosis (more than 1000×10^9/L) uncontrolled by therapy.
- Megakaryocyte proliferation in sheets or clusters in association with fibrosis.

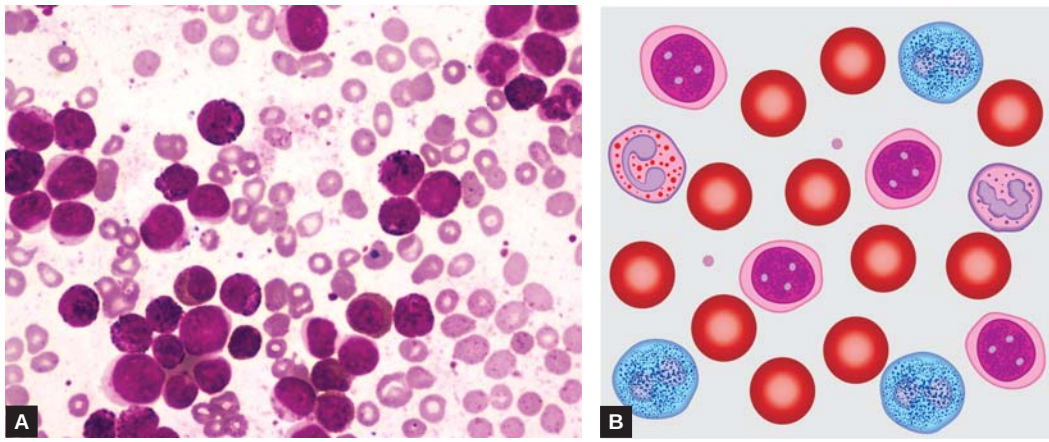
- Additional clonal cytogenetic/chromosomal abnormalities may appear (like trisomy 8, isochromosome 17q or duplication of the Ph chromosome).
- Persistent or increasing splenomegaly unresponsive to therapy.

Blast Phase/crisis (BP)

- Blood picture resembles acute leukemia.
- This final phase lasts for a few weeks to months and has a poor prognosis.
- ***Peripheral smear*** (Figs 26.6A and B)
  - Blasts constitute 20% or more and may be of the myeloid (70% cases) or lymphoid type (30% cases).
  - Basophils may increase to 20% or more.
  - Thrombocytopenia may result in bleeding episodes.
- ***Bone marrow*** fibrosis develops and may cause marrow failure.
- ***Lymphadenopathy*** may appear.

Treatment

- The drug imatinib is a BCR-ABL inhibitor, which blocks the adenosine triphosphate (ATP)-binding site on the BCR-ABL tyrosine kinase, thereby inactivating it. Imatinib markedly reduces the number of BCR-ABL-positive cells and reduces the blood cell counts and the risk of transformation to the accelerated phase and blast crisis.



Figs 26.6A and B: Peripheral blood picture in blast phase of chronic myeloid leukemia showing numerous blasts (20% or more) and striking basophilia

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- Allogeneic bone marrow transplantation is the treatment of choice and is curative in majority of patients in the stable phase.

Prognosis: The prognosis is poor in patients with accelerated phase or blast crisis.

MYELODYSPLASTIC/MYELOPROLIFERATIVE NEOPLASMS

In the present WHO classification of tumors of hematopoietic tissue, the following tumors are categorized under the myelodysplastic/myeloproliferative neoplasms.

Chronic Myelomonocytic Leukemia (CMML)

Definition: It is a clonal hematopoietic malignancy characterized by features of both a myeloproliferative neoplasm and a myelodysplastic syndrome.

- It is seen in the middle aged or elderly and usually affects males.
- Onset is insidious and presents with splenomegaly (in about 40-50% of cases), anemia, fever, bleeding and infection.
- It may transform into AML.
- Marrow cells do not show Ph chromosome.
- *Peripheral smear*
  - **RBCs:** Show variable anemia.
  - **WBCs:** Total leukocyte count may be markedly increased. **Absolute monocytosis** (more than 1×10^9/L) is a prominent feature.
  - **Platelets:** Thrombocytopenia is common (unlike classical CML).
- *Bone marrow*
  - Hypercellular with granulocytic and monocytic hyperplasia.
  - Features of myelodysplasia of hematopoietic cell lines are evident.

Juvenile Myelomonocytic Leukemia (JMML)

Definition: It is a clonal hematopoietic disorder of childhood characterized by proliferation mainly of the granulocytic and monocytic lineage.

- It is a rare disease found in children below 14 years with 75% occurring in children less than 3 years of age.
- *Peripheral smear:*
  - **RBCs:** Show normocytic normochromic anemia.
  - **WBCs:** Total leukocyte count is higher (may reach 100×10^9/L) but unlike CML, myelocytes are not the predominant cells. Blasts are less than 20% and monocytosis is more than 1×10^9/L.
  - **Platelets:** Decreased (thrombocytopenia).
- **Fetal hemoglobin (HbF):** Increased in 70% of cases.

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Clinical features

- The symptoms of anemia and splenomegaly are usually present. Lymphadenopathy may also be present.
- These patients have neither Ph chromosome nor BCR-ABL.

Prognosis: Fatal.

SUMMARY

- Chronic myeloid leukemia (CML) is an acquired clonal myeloproliferative neoplasm of the pluripotent hematopoietic stem cell.
- It is characterized by anemia, extreme leukocytosis, granulocytic immaturity, basophilia, thrombocytosis and splenomegaly.
- It is distinguished from other chronic myeloproliferative neoplasm by the presence of Philadelphia chromosome in more than 90% of cases.
- Philadelphia chromosome is a balanced reciprocal translocation between long arm of chromosome 9 and chromosome 22. The resulting BCR-ABL fusion gene is responsible for autophosphorylation and constitutive tyrosine kinase activity. The net outcome is neoplastic cell proliferation of mainly myeloid precursors and inhibition of apoptosis.
- LAP (NAP) score is markedly reduced.
- There are three phases in CML, namely chronic stable phase, accelerated phase and blast phase.
- In the present WHO classification of tumors of hematopoietic tissue, chronic myelomonocytic leukemia and juvenile myelomonocytic leukemias are categorized under the myelodysplastic/myeloproliferative neoplasms.

SELF-ASSESSMENT EXERCISES

I. Essays

1. Discuss chronic myeloid leukemia
2. Laboratory findings in chronic myeloid leukemia

II. Short Notes

1. Philadelphia chromosome
2. Peripheral blood picture in various phases of chronic myeloid leukemia
3. Prognostic factors of CML

III. Multiple Choice Questions

1. **The following statements is NOT TRUE with respect to Philadelphia chromosome**
   A. It is an acquired chromosomal abnormality present in all proliferating hematopoietic stem cells
   B. It is a balanced reciprocal translocation between long arm of chromosome 9 and chromosome 22
   C. It produces a new chimeric (fusion) gene called BCR-ABL
   D. Its presence is a bad prognostic indicator

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2. **The most common translocation found in patients with chronic myelogenous leukemia is**
   A. t(8;14)
   B. t(8;21)
   C. t(9;22)
   D. t(15;17)
3. **The hematological disorder which shows BCR-ABL fusion gene is**
   A. Polycythemia vera
   B. Erythroleukemia
   C. Chronic myeloid leukemia
   D. von Willebrand disease
4. **The cytochemical stain which helps to differentiate leukemoid reaction from chronic myeloid leukemia is**
   A. Sudan Black
   B. Periodic acid Schiff
   C. Nonspecific esterase
   D. Leukocyte alkaline phosphatase
5. **The feature NOT seen in chronic myeloid leukemia is**
   A. Leukocytosis
   B. Thrombocytosis
   C. Absence of basophils
   D. Decreased leukocyte alkaline phosphatase
6. **The type of leukemia associated with basophilic leukocytosis is**
   A. Acute myeloid leukemia
   B. Acute lymphoid leukemia
   C. Chronic myeloid leukemia
   D. Chronic lymphoid leukemia
7. **The following is NOT a phase found in chronic myeloid leukemia**
   A. Blast phase
   B. Accelerated phase
   C. Stable phase
   D. Unstable phase

ANSWERS

1. D **2.** C **3.** C **4.** D **5.** C **6.** C **7.** D

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Chapter 27 Chronic Lymphocytic Leukemia and Other Lymphoid Leukemias

Chapter Outline

- Chronic Lymphocytic Leukemia
- Prolymphocytic Leukemia
- Hairy Cell Leukemia

CHRONIC LYMPHOCYTIC LEUKEMIA

Definition

Chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL) is tumor composed of monomorphic small B lymphocytes in the peripheral blood, bone marrow and lymphoid organs (spleen and lymph nodes).

WHO considers both CLL and SLL as a single entity with different presentations. **Small lymphocytic lymphoma** (SLL) is considered as a tissue equivalent of chronic lymphocytic leukemia (CLL). The CLL/SLL tumor cells coexpress CD5 and CD23.

Etiology and Pathogenesis

- ***Environmental factors***: Different environmental factors have been suggested but none are proved to have a significant role.
- ***Hereditary factors*:** Families with higher risk of CLL or other lymphoid neoplasms have been observed.

Cytogenetic Abnormalities

Chromosomal translocations are rarely observed in both CLL and SLL. The common mutations associated are deletions of 13q14.3, 11q22-23, and 17p13. About 20% of CLL show trisomy 12.

Clinical Features

- ***Age:*** Most of the patients at the time of diagnosis are between **50-60 years** of age.
- ***Sex:*** More common in males than in females with a ratio of 2:1.
- ***Asymptomatic:*** About 25% of patients are often asymptomatic and are detected either because of nonspecific symptoms or routine blood examination for some other disease.
- ***Nonspecific symptoms:*** This includes fatigue, loss of weight and anorexia.

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- ***Generalized lymphadenopathy:*** Initially the cervical lymph nodes are enlarged and in later stages there may be generalized lymphadenopathy. Involved nodes are rubbery, discrete, non-tender, small and mobile.
- ***Splenomegaly and hepatomegaly:*** This is observed in very few cases. Infiltration by CLL cells is seen in the splenic white and red pulp and in hepatic portal tracts resulting in enlargement of both organs.
- ***Immunological defects:*** CLL/SLL disrupts normal immune function, the mechanisms of which are not known. The pathogenic IgGs are produced by non-neoplastic, self-reactive B cells rather than tumor cells resulting in the following:
  - **Immune deficiency:** Hypogammaglobulinemia is common and is responsible for increased risk of opportunistic infections.
  - **Autoimmunity:** Patients may develop systemic autoimmune diseases, especially hemolytic anemia or thrombocytopenia due to autoantibodies.

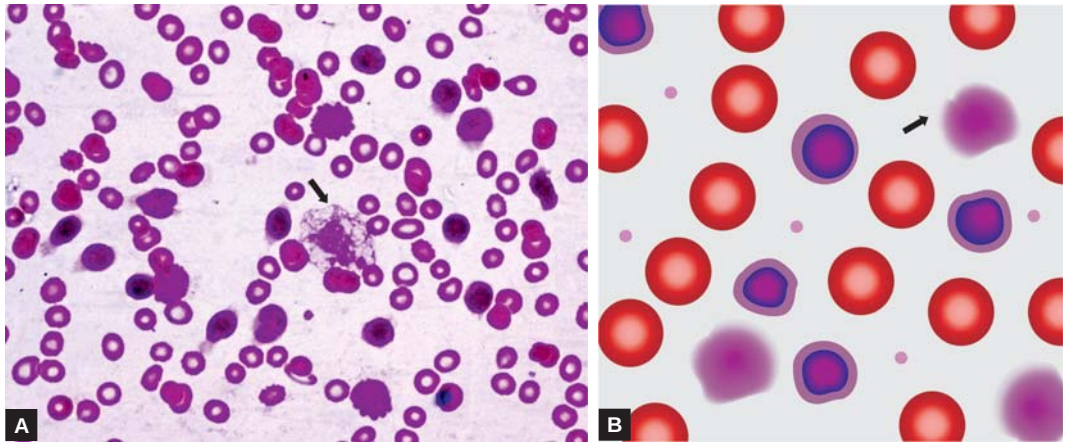
Laboratory Findings

Peripheral Blood

- **Hemoglobin**: Usually below 13 gm/dL and as the disease progresses, it may decrease below 10 gm/dL. This is due to marrow failure and associated autoimmune hemolysis may also be contributory, when present.
- **Peripheral smear (Figs 27.1A and B)**
  - **RBCs:** Show normocytic normochromic anemia or rarely hemolytic blood picture.
  - **WBCs:** Total leukocyte count is increased and varies from 20-50 × 10^9/L.
    - **Differential Leukocyte Count:**
      Lymphocytosis is the characteristic feature and absolute lymphocyte count should be more than 5 × 10^9/L. Usually absolute lymphocyte count above 10 × 10^9/L is common at the time of diagnosis.
      Lymphocytes constitute more than 50% of the white cells and may reach up to 90-98% with resultant neutropenia (lymphocytes 70-98% and polymorphs 2-30%).
      Lymphocytes are of mature type in majority of the cases. Morphologically, they are small with scant or a very thin rim of blue cytoplasm. The **nuclei** are usually round with regularly clumped coarse chromatin **("soccer ball"/block-type chromatin).** Few cells may have notching of the nuclei. Nucleoli are inconspicuous or absent.
      Smudge cells: Smudge cells or basket cells are disintegrated lymphocytes and represent the spread out nuclear material observed in the peripheral blood film. They are due to rupture of the neoplastic lymphoid cells while making the peripheral smear due to its fragile nature.
  - **Platelets:** Initially the platelet count is normal. The bone marrow infiltration as well as autoimmune destruction of platelets associated with hypersplenism may result in decreased platelet count and a count below 100 × 10^9/L is associated with a worse prognosis.

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Figs 27.1A and B: Peripheral blood smear in chronic lymphocytic leukemia showing numerous small lymphocytes and few smudge cells (arrow)

Bone Marrow

- **Cellularity:** Bone marrow is involved in all cases of CLL and its infiltration by mature lymphocytes results in hypercellular marrow. The infiltration in the trephine biopsy may be in the form of interstitial infiltrates and/or non-paratrabecular aggregates of small lymphocytes.
- **Erythropoiesis:** Erythropoiesis is normal. Patients are prone to develop autoimmune diseases, most commonly directed against red cells or platelets. Such cases which result in hemolytic anemia show normoblastic erythroid hyperplasia.
- **Myelopoiesis:** Myelopoiesis is normal in the initial stages of the disease.
- **Megakaryopoiesis:** Megakaryopoiesis is within normal limits.

  As the neoplastic lymphocytes increase in number, they replace the normal cells of erythroid, myeloid and megakaryocytic series in the bone marrow. This results in anemia, neutropenia and thrombocytopenia and indicates that the disease is advancing.

Antiglobulin (Coombs) Test

About 15 to 20% of patients manifest autoimmune hemolytic anemia and have positive direct Coombs test.

Immunophenotype

The tumor cells in CLL/SLL express the pan-B cell markers CD19 and CD20. B cell CLL is characterized by aberrant expression of T cell antigen CD5 (found only in a small subset of normal B cells). There is also weak expression of monoclonal surface immunoglobulin with **κ or λ light chains.**

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Lymph Node

Affected lymph nodes are enlarged and the cut surface is homogeneous white. They show loss of normal architecture due to diffuse infiltration by monomorphic, small, round lymphocytes measuring 6 to 12 μm in diameter. These lymphocytes have round to slightly irregular nuclei having condensed coarse chromatin and scanty cytoplasm.

There are small, loose nodular aggregates of prolymphocyte-appearing, mitotically active medium to large-sized lymphocytes found scattered in the lymphnode. Their nuclei have dispersed chromatin with distinct nucleoli. These aggregates are known as **proliferation centers or pseudo follicles or growth centers and when found are pathognomonic for CLL/SLL**. Compared to the other region of the lymphnode, the pseudofollicles appear pale because of the greater distances between nuclei of their constituent "large prolymphocytes".

Cells of CLL are relatively resistant to apoptosis. They express high levels of antiapoptotic protein BCL-2.

Course and Prognosis

Median survival rate is 4 to 6 years. Course is variable and depends on:

- Clinical stage of disease.
- Plasma β2 microglobulin level: High levels are associated with a poor prognosis.
- Presence of deletions of 11q and 17p: Worse prognosis.
- Bone marrow trephine biopsy: Pattern of marrow infiltration. Nodular and interstitial pattern of infiltration has better prognosis than mixed and diffuse types.
- Lack of somatic hypermutation: Worse prognosis.

Progression and Transformation of CLL

CLL/SLL may transform to more aggressive tumors. These transformations are probably due to additional mutations resulting in rapid growth and are indicative of poor prognosis.

B cell Prolymphocytic Transformation

Transformation to B cell prolymphocytic leukemia is extremely rare. It is characterized by:

- Appearance of "prolymphocytes" in the peripheral blood (>10%). These prolymphocytes are large cells with a single prominent centrally placed nucleolus.
- Worsening of cytopenias.
- Increasing splenomegaly.

Transformation to Diffuse Large B cell Lymphoma (Richter syndrome)

It is found in 5 to 10% of patients and is characterized by the development of a rapidly enlarging mass within a lymph node or the spleen.

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PROLYMPHOCYTIC LEUKEMIA

Prolymphocytic leukemia is a rare neoplasm of either B or T cell type.

B cell Prolymphocytic Leukemia

Definition

B cell prolymphocytic leukemia is a rare neoplasm of B prolymphocytes affecting peripheral blood, bone marrow and spleen.

Clinical Features

Age and Sex: Most of the patients are above 60 years and equally affect both sexes.

Spleen: Splenic involvement causes moderate to marked splenomegaly.

Lymphadenopathy: Cervical lymphadenopathy may be minimal.

Laboratory Findings

Peripheral blood

- **Total leukocyte count:** Increased and ranges from 30 to 200 × 10^9/L. It is usually higher than that found in CLL.
- **Hemoglobin:** Reduced and varies from 7 to 12 gm/dL.
- **Platelet count:** Reduced and in majority of patients it is less than 100x10^9/L.
- **Peripheral smear**
  - **RBCs:** Show normocytic normochromic anemia.
  - **WBCs:** Shows prolymphocytes which constitute more than 55% of DLC. Prolymphocytes are medium-sized cells (twice the size of a lymphocyte) with round nucleus having condensed nuclear chromatin with prominent single central nucleolus; relatively small amount of faintly basophilic cytoplasm.
  - **Platelets:** Reduced.

Bone marrow

- **Cellularity: Hypercellular** due to infiltration by prolymphocytes which constitute more than 55% of the marrow cells.
- **Erythropoiesis: Diminished** and results in anemia.
- **Myelopoiesis: Diminished** and causes neutropenia.
- **Megakaryopoiesis: Diminished** and results in thrombocytopenia.

Spleen

Both red and white pulp shows **infiltration by prolymphocytes**.

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Immunophenotype

B cell markers CD19, CD22 are positive. Specific markers FMC7, CD79b and SmIg are strongly positive.

Cytogenetics

Del (17p) is detected in 50% of patients.

Prognosis

Responds poorly to therapies for CLL.

HAIRY CELL LEUKEMIA

Definition

Hairy cell leukemia **(HCL)** is an uncommon indolent neoplasm of small mature B cells having abundant cytoplasm with fine hairy projections.
The term **Hairy cell leukemia** is derived from the appearance of **fine hair-like cytoplasmic projections** on the leukemic cells, when viewed under the **phase-contrast microscope.**

Cell of Origin

Late, activated memory B lymphocytes.

Site Involved

This neoplasm involves peripheral blood, bone marrow, spleen and liver.

Laboratory Findings

Peripheral Blood

- **Hemoglobin: Decreased** and is usually less than 8 gm/dL.
- **Total leukocyte count:** More than three fourths of the patients demonstrate **leukopenia** (with neutropenia); few patients may have mild leukocytosis.
- **Platelet count:** It is usually **less** than 50×10^9/L.
- **Peripheral smear:** Shows **pancytopenia** in majority of cases.
  - **RBCs:** Show normocytic normochromic picture
  - **WBCs:** Show leukopenia with few **hairy cells.** Characteristics of hairy cells are:
    Size: Hairy cells are **medium-sized** (1.5 to 2 times the size of normal lymphocyte) lymphoid cells with size of 10 to 15 μm.
    Cytoplasm: It is **abundant and pale blue.** The cytoplasmic borders are indistinct and appear frayed. The characteristic fine, filamentous (fine thread-like or bleb-like) **cytoplasmic projections resembling hair** are present in only a subset of cells.

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Nuclei: They are oval to **reniform** (bean-shaped), centrally placed and have smooth nuclear membranes. The chromatin is granular and is intermediate between that of a small lymphocyte and a lymphoblast. Nucleoli are either absent or inconspicuous.

Cytochemistry: Tartrate resistant acid phosphatase **(TRAP) positivity** in the cytoplasm is the **characteristic** feature of HCL.

- **Platelets:** They are reduced in number.

Bone Marrow

Aspiration: Marrow aspirate often yields a **dry tap,** because hairy cells are enmeshed between the reticulin fibrils and are difficult to aspirate. Hairy cells in the marrow are morphologically similar to those seen in peripheral blood smear. There is moderate to marked **reduction in myeloid, erythroid and megakaryocytic** cell lines.

Trephine biopsy: The diagnosis is best made on trephine biopsy. The marrow shows interstitial or patchy infiltration with some preservation of fat and hematopoietic elements. The abundant, pale cytoplasm and prominent cell border of neoplastic cells may impart a **"fried egg" or "honeycomb" appearance** (which is not found in any other lymphoproliferative disorder). **Reticulin stain** on the marrow biopsy shows marked **increase of** thin **reticulin fibers** surrounding individual neoplastic cells.

Spleen

Spleen is **enlarged** due to leukemic infiltrate of the red pulp the white pulp show atrophy. **Sinuses** of spleen are characteristically lined by **hairy cells** and **grossly** impart a **beefy red** appearance.

Immunophenotype and Molecular Characteristics

Hairy cells usually express the **CD20, CD22, CD11c and CD25** (the IL-2 receptor α-chain) **positivity**. Annexin A 1 is the most specific marker of hairy cell leukemia.

Clinical Features

- Hairy cell leukemia affects **middle-aged to elderly men**, with a male-to-female ratio of 5:1.
- Clinical features are mainly due to leukemic infiltration of bone marrow, liver and spleen.
  - ***Massive Splenomegaly*** is the most common finding, hepatomegaly is less common and lymphadenopathy is distinctly rare.
  - ***Pancytopenia*** is due to marrow failure and splenic sequestration. It is found in more than 50% of cases. About one third of patients present with infections especially with atypical mycobacteria, which may be due to monocytopenia and is characteristic of HCL.

Prognosis: HCL tends to follow an indolent course and prognosis is **excellent.**

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SUMMARY

- Small lymphocytic lymphoma/chronic lymphocytic leukemia are seen in older adults with involvement of bone marrow, lymph node, spleen and liver.
- CLL is characterized by accumulation of lymphoid cells in peripheral blood and bone marrow. When it involves lymphoid organs, it is known as small lymphocytic lymphoma.
- The peripheral blood shows lymphocytosis with smudge cells.
- The small lymphocytes are mature cells having scanty cytoplasm, coarse chromatin and inconspicuous nucleoli.
- Prolymphocytic leukemia is a rare neoplasm of either B or T cell type.
- Hairy cell leukemia is an uncommon neoplasm of B cells. These tumor cells have cytoplasm with hairy projections and show TRAP positivity.

SELF-ASSESSMENT EXERCISES

I. Short Notes

1. Peripheral smear findings in chronic lymphocytic leukemia
2. Laboratory findings in chronic lymphocytic leukemia
3. Transformation in chronic lymphocytic leukemia
4. Hematological findings in chronic lymphocytic leukemia
5. Hairy cell leukemia

II. Multiple Choice Questions

1. **Transformation of CLL to diffuse large B cell lymphoma is known as**
   A. Lymphoma spill over
   B. Richter syndrome
   C. Aggressive transformation
   D. Prolymphocytic transformation
2. **The smudge cells in the peripheral smear are seen in**
   A. ALL
   B. AML
   C. CLL
   D. CML
3. **Majority of chronic lymphocytic leukemia arise from**
   A. B cell
   B. T cell
   C. Null cell
   D. NK cell
4. **The round nuclei with block-type chromatin or soccer ball chromatin are seen in cells of**
   A. ALL
   B. AML
   C. CLL
   D. CML

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5. **The cell of origin of prolymphocytic leukemia is**
   A. B cell
   B. T cell
   C. NK cell
   D. B/T cell
6. **Immunophenotype marker for B cell prolymphocytic leukemia is**
   A. CD2
   B. CD5
   C. CD8
   D. CD22
7. **The following statement is NOT true about hairy cell leukemia**
   A. It is a T cell neoplasm.
   B. The cells have fine, filamentous cytoplasmic projections.
   C. Tartrate resistant acid phosphatase (TRAP) positivity is helpful diagnostic test.
   D. Trephine biopsy of marrow shows tumor cells with fried egg appearance.
8. **"Fried egg" or "honeycomb" appearance in bone marrow biopsy is characteristically seen in**
   A. Hodgkin lymphoma
   B. Burkitt lymphoma
   C. Mycosis fungoides
   D. Hairy cell leukemia
9. **Tartrate resistant acid phosphatase positivity in the cytoplasm is the characteristic feature of**
   A. Hairy cell leukemia
   B. Chronic myeloid leukemia
   C. Chronic lymphocytic leukemia
   D. Mycosis fungoides

ANSWERS

1. B	**2.** C	**3.** A	**4.** C	**5.** D
6. D	**7.** A	**8.** D	**9.** A	

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Chapter 28

Plasma Cell Neoplasms

Chapter Outline

- Introduction
- Plasma Cell Myeloma
- Plasmacytoma
- Immunoglobulin Deposition Diseases
- Monoclonal Gammopathy of Uncertain Significance
- Osteosclerotic Myeloma

INTRODUCTION

Plasma cells are the immunoglobulin-producing cells and are normally found in the bone marrow. Normally serum immunoglobulins are polyclonal and are produced by many different clones of plasma cells.

A gammopathy refers to over-production of one more types or classes of immunoglobulin. It may be polyclonal which is found with acute or chronic inflammation such as infection,sarcoidosis, autoimmune disorders or some malignancies. Monoclonal is characterized by increase in a single immunoglobulin class which may occur in association with normal or reduced level of other immunoglobulins. Such monoclonal proteins, also called M-proteins, paraproteins or monoclonal gammopathies, occur as a feature of plasma cell neoplasms, lymphoma and amyloidosis, in connective tissue disease such as rheumatoid arthritis, in infection such as HIV and in solid tumors. In addition they may be present with no underlying disease.

Definition

Plasma cell neoplasms are a group of B cell neoplasms associated with the monoclonal proliferation (single clone) of immunoglobulin-secreting plasma cells. These are also known as dyscrasias.

Characteristics of Plasma Cell Neoplasms

The single clone of neoplastic plasma cells typically secrete a single homogeneous (monoclonal) complete immunoglobulin (Ig) or Ig fragment. The abnormal monoclonal Ig proteins identified in the serum as a band in protein electrophoresis are termed as M component (M for myeloma)/ myeloma proteins (M-protein) or paraproteins. Thus, these disorders are known as paraproteinemias (monoclonal gammopathies, dysproteinemias).

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- **Complete M proteins** have high molecular weight (160,000 or more) and are thus found only in the plasma and extracellular fluid and are **not excreted in the urine** unless there is glomerular damage.
- **Normal plasma cells** secrete **equal quantities of heavy and light chains.** But the neoplastic plasma cells frequently synthesize excess of light (L) or heavy (H) chains along with complete Igs. Rarely, only light chains or heavy chains are produced.
- The **excess of free light chains** are small with low molecular weight and therefore, excreted in the urine. They are known as **Bence-Jones proteins.**

Classification of Plasma Cell Neoplasms (Table 28.1)

Table 28.1: Classification of plasma cell neoplasms (WHO 2008)

Plasma cell myeloma
- ***Variants***
  - Asymptomatic (smoldering) myeloma
  - Nonsecretory myeloma
  - Plasma cell leukemia

Plasmacytoma
- Solitary plasmacytoma of bone
- Extraosseous (extramedullary) plasmacytoma

Immunoglobulin deposition diseases
- Primary amyloidosis
- Systemic light and heavy chain deposition diseases

Monoclonal gammopathy of undetermined significance (MGUS)

Osteosclerotic myeloma (POEMS syndrome)

PLASMA CELL MYELOMA

Definition

Plasma cell myeloma (multiple myeloma) is a malignant, multifocal plasma cell neoplasm of the bone marrow associated with an M-protein in the serum and/or urine.

It is the most common and important monoclonal gammopathy and usually presents as multiple tumor masses throughout the skeletal system.

Etiology and Pathogenesis

Risk factors: The cause of multiple myeloma is unclear and few risk factors have been observed.

- ***Genetic predisposition***: Increased incidence of multiple myeloma in first-degree relatives and in identical twins has been reported.
- ***Exposure to ionizing radiation***: Long-term survivors of atomic bomb explosions, radiologists, nuclear industry workers, uranium miners and radium dial painters have an increased incidence of multiple myeloma.
- ***Chronic antigenic stimulation***: Few cases of multiple myeloma have been found in association with chronic infections (HIV and chronic osteomyelitis) and chronic inflammatory disorders (e.g. rheumatoid arthritis). A two-hit hypothesis has been suggested.

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First hit of antigenic stimulation causing reactive, polyclonal proliferation of B lymphocytes; and the second mutagenic hit results in malignant monoclonal proliferation.

- ***Exposure to chemicals*** like benzene, other organic solvents, herbicides and insecticides are also some of the other risk factors.

Cell of Origin

Myeloma arises from the **post-germinal center B cell** in the bone marrow which shows differentiation into plasma cell. It appears that post-germinal center B cells undergo somatic hypermutation and further oncogenic events that convert these cells into plasmablast/abnormal plasma cells. These tumor cells are called myeloma cells.

Cytogenetic Abnormalities

Myeloma is molecularly quite heterogeneous and is associated with diverse chromosomal aberrations. These include translocations, deletions and dysregulation of cyclins, etc.

Pathological Features

The major pathologic features of multiple myeloma are:

- **Proliferation of plasmablasts/plasma cells in the bone marrow:** The proliferation and survival of myeloma cells are dependent on several cytokines, most important being IL-6, which is produced by both myeloma cells as well as marrow stromal cells. Serum IL-6 levels correlate with activity of the disease and prognosis.
- **Multiple lytic lesions in bones and hypercalcemia (Fig. 28.1):** Bone destruction resulting in multiple lytic bone lesions, hypercalcemia and pathologic fractures are prominent features of multiple myeloma.
  - In MM, the myeloma cells adhere to bone marrow stroma. Interaction of myeloma cells with stromal cells stimulates excessive production of cytokines and inflammatory proteins. These include interleukins IL-6, IL-1, tumor necrosis factor (TNF) and macrophage inflammatory protein 1α (MIP-1α). These chemicals mediate bone destruction by activating bone resorbing osteoclasts and hence are called osteoclast activating factors (OAFs).
  - OAFs also indirectly induce osteoclast formation by stimulating marrow stromal cells to express increased levels of RANKL (RANK ligand) on their surface. RANKL binds to the transmembrane RANK receptor (receptor activator for NF-κB) on osteoclast precursors and activates the transcription factor NF-κB. This results in differentiation and maturation of the osteoclast progenitor cells into osteoclasts. Thus abnormal osteoclast activity induced by tumor cells and inhibition of osteoblast differentiation causes erosion of bone.
  - Myeloma cells also produce MIP-1(α/β) and directly activate the osteoclast precursors.
  - A naturally occurring decoy receptor osteoprotegrin (OPG) secreted by osteoblasts normally blocks RANKL interaction with RANK. Myeloma cells degrade OPG and are responsible for reduced OPG level. There is an inverse correlation between OPG levels and the degree of skeletal destruction. The major imbalance between RANKL (highly increased

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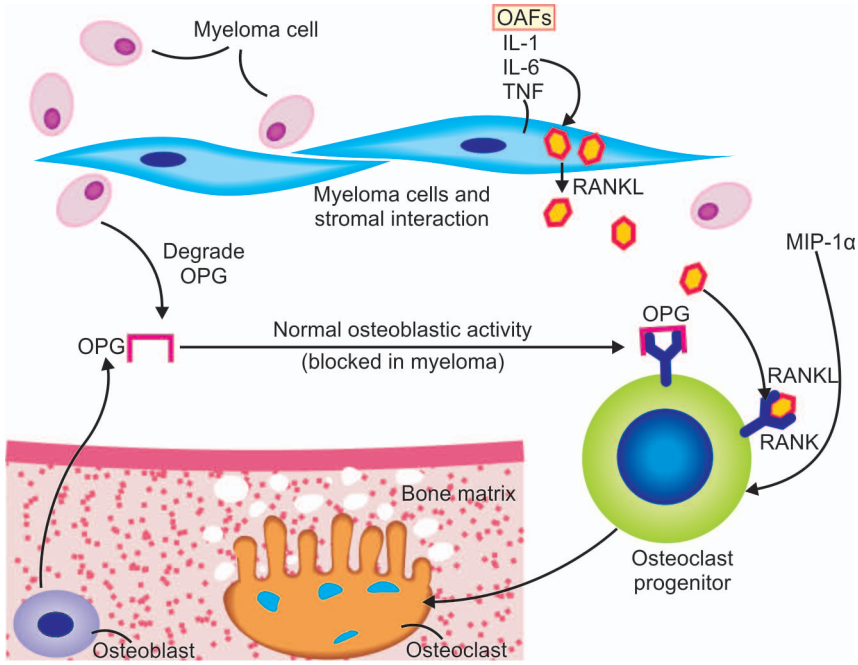


Fig. 28.1: Pathogenesis of bone resorption in myeloma. OPG = Osteoprotegrin; OAFs = Osteoclast activating factors; RANK = Receptor activator of nuclear factor κB; RANKL = RANK ligand; MIP-1α = Macrophage inflammatory protein 1-α

activity) and OPG (markedly decreased activity) switches the balance totally towards bone destruction.

- Tumor cells also produce modulators of WNT, which inhibit osteoblast activity.

- **Monoclonal gammopathy:** Most myelomas secrete monoclonal immunoglobulins or light chains. They may secrete excess light chains due to unbalanced synthesis of heavy and light chains. The monoclonal light chains are rapidly filtered through glomeruli and appear in the urine as Bence-Jones proteins.

Diagnostic criteria for plasma cell myeloma: They are shown in Table 28.2.

Table 28.2: Diagnostic Criteria (Durie and Salmon)

Major criteria
- Plasmacytoma on tissue biopsy
- Bone marrow plasmacytosis > 30% plasma cells
- Monoclonal globulin spike. IgG > 35 g/L, or IgA >20 g/L
  Light chain excretion on urine electrophoresis ≥1 g/24 hours

Minor criteria
- BM plasmacytosis, 10-30% plasma cells
- Monoclonal globulin spike, IgG < 35 g/L or IgA < 20 g/L
- Lytic bone lesions
- Normal IgM < 500 mg/L, IgA < 1 g/L or IgG < 6 g/L

Criteria for MM
- 1 major +1 minor criterion or
- 3 minor criteria that must include 1 and 2 of minor criteria

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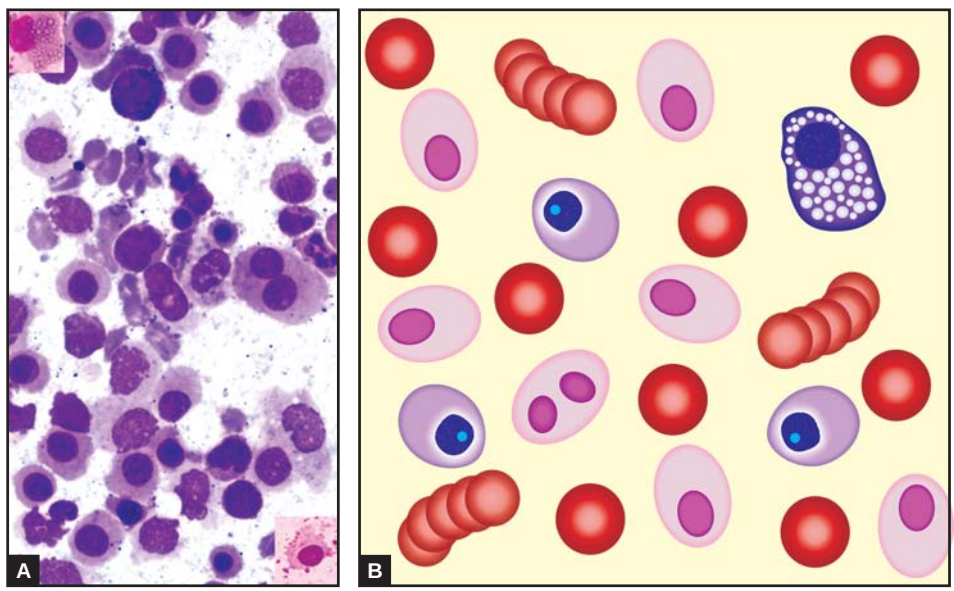
Laboratory Findings

Peripheral Blood

- **Hemoglobin:** It is **decreased** and usually between 6 and 10 gm/dL.
- **Peripheral smear**
  - **RBCs:** Usually normocytic normochromic anemia occurs in nearly all patients. There is a tendency for the red cells to adhere to one another and form columns in the blood film referred to as **rouleaux formation** (see Fig. 42.6). This is due to increased immunoglobulins. Rouleaux formation is characteristic but not diagnostic, as it may be seen in any condition with elevated immunoglobulins.
  - **WBCs:** Total and differential leukocyte count is **normal.** In terminal stages of the disease, few plasma cells may be observed in the peripheral blood. If number of **plasma cells is higher** than 2×10^9/L, it is labeled as **plasma cell leukemia**.
  - **Platelets**: Platelet count is usually within **normal** limits.
- **ESR: Increased** because of rouleaux formation due to high immunoglobulin levels.
- **Bleeding time**: Platelets may get coated by M-component which impairs platelet aggregation resulting in **increased** bleeding time.

Bone Marrow

- **Cellularity**: Bone marrow is **hypercellular**, as a result of increased number of plasma cells and **myeloma plasma cells** (neoplastic plasma cells). These cells may infiltrate the interstitium or may be present in diffuse sheets or nodular aggregates replacing the normal hematopoietic elements (Figs 28.2A and B).



Figs 28.2A and B: Bone marrow aspirate in multiple myeloma. Inset in A shows flame cell (left lower corner) and mott cell (right upper corner)

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- Increased number of **myeloma plasma cells more than 30%** of the cellularity is diagnostic. When their number is less than 10%, demonstration of their monoclonal (κ or λ) nature is highly suggestive of myeloma. Myeloma plasma cells are neoplastic plasma cells that vary from mature plasma cells, plasmablasts (immature) and pleomorphic plasma cells.
  - **Myeloma plasma cells (mature type)** are large usually oval with round to oval eccentrically placed nucleus and abundant pale blue cytoplasm. The nucleus may show "spoke wheel" or "clock-face" chromatin without nucleoli. A perinuclear hof (clearing) due to a prominent Golgi apparatus is present. **Few** of these cells may show **binucleation** and **multinucleation.**
  - **Plasmablastic (immature) myeloma cells** have higher nuclear/cytoplasmic ratio and round nucleus with vesicular/dispersed chromatin often with prominent single nucleolus. Presence of these cells is associated with a worse prognosis.
  - **Pleomorphic plasma cells** are multinucleated or polylobated and they may be prominent in some cases. Since nuclear immaturity and pleomorphism are rare in reactive plasma cells, they are reliable indicator of malignant plasma cells.
- **Other morphological findings:** The cytoplasm of myeloma cells has abundant endoplasmic reticulum, which may contain condensed or crystallized immunoglobulin. This may produce distinct morphological patterns (Fig. 28.3).

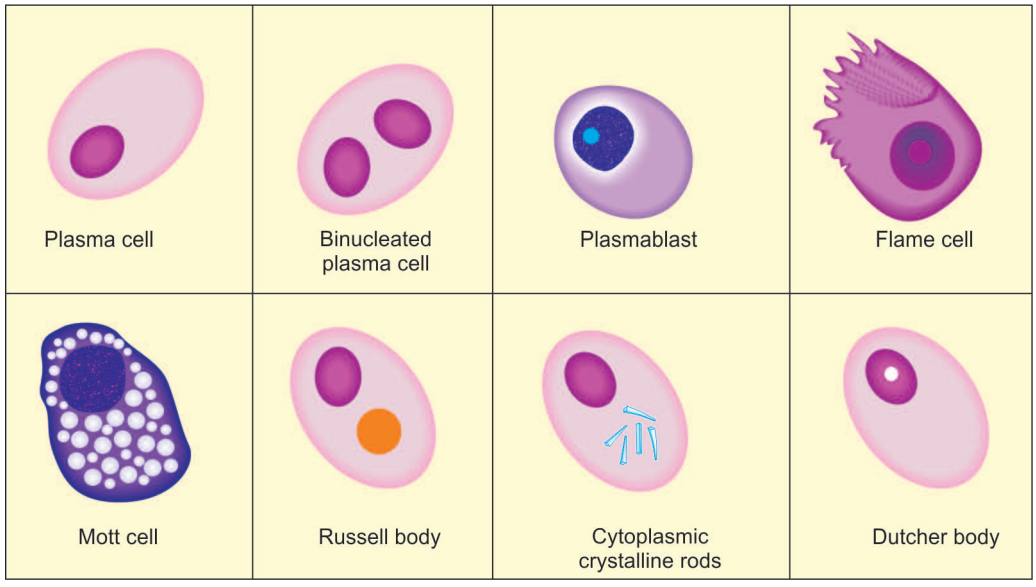


Fig. 28.3: Morphologic findings of myeloma cell based on cytoplasmic/nuclear features

  - **Flame cells:** These cells show peripheral fiery red (vermilion staining) glycogen-rich immunoglobulin A and have irregularly shaped or flame-like cytoplasm and hence the name.
  - **Grape (morula/mott) cells:** These cells have multiple bluish-white, grapelike rounded cytoplasmic droplets.

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  - **Russell bodies:** These are globular, eosinophilic (cherry red), refractile inclusions in the cytoplasm.
  - **Cells with a variety of other inclusions:** These include **fibrils (Gaucher-like cells, thesaurocytes), crystalline rods and globules.**
  - **Dutcher bodies:** They represent intranuclear pseudo inclusions.
- **Erythropoiesis:** It is diminished and is normoblastic.
- **Myelopoiesis:** It shows normal maturation.
- **Megakaryopoiesis:** Normal.

As the tumor progresses, it may compromise erythroid, myeloid and megakaryocytic progenitors resulting in pancytopenia.

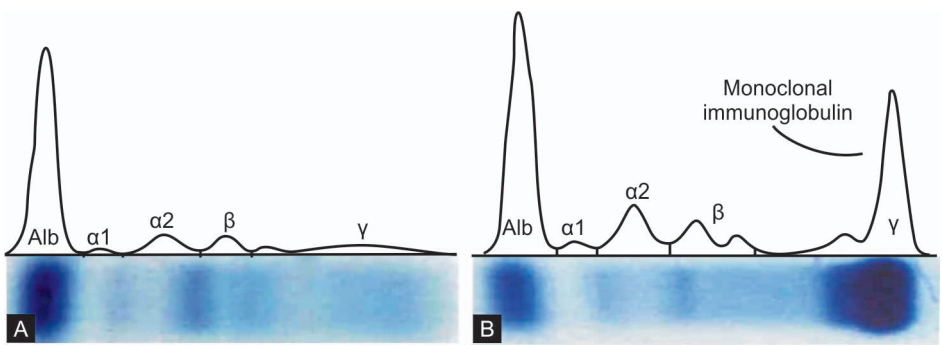
Urine: It may show Bence Jones protein (refer page 440)

Serum Findings

- **Serum β_2 microglobulin**: It is a useful **prognostic marker** and **high values signify poor prognosis.**
- **Hypercalcemia:** It is due to extensive osteolytic lesions and osteoporosis and there are also increased levels of serum phosphate and serum alkaline phosphatase.
- **Blood urea, serum creatinine and uric acid:** They are often raised when there is impairment of renal function. This is associated with reduced creatinine clearance.
- **Serum albumin:** Decreases in advance stages of the disease.

Electrophoretic Studies on Serum and Urine

Electrophoretic studies reveal raised levels of immunoglobulins in the blood and/or light chains (Bence Jones proteins) in the urine. The monoclonal immunoglobulin ("M protein") is identified as abnormal protein "spikes" in serum or urine electrophoresis and is then further characterized by immunofixation (Figs 28.4A and B). The most common M protein is IgG type, followed by IgA.



Figs 28.4A and B: Serum electrophoresis showing normal pattern: (A) and monoclonal immunoglobulin ("M Band") in multiple myeloma (B)

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Morphology of Organs Involved

- **Bone:** Multiple myeloma usually presents as **destructive lytic lesions** in the axial skeleton**.** The most commonly involved bones are the vertebral column and flat bones like skull, ribs and pelvis. Destruction of cortex often results in pathologic fractures; usually of the vertebra. Radiographically, the characteristic lesions appear as **punched-out** lytic lesions**,** usually 1 to 4 cm in diameter (Fig. 28.5). It may also result in generalized osteoporosis or fractures at other sites.



Fig. 28.5: Skull X-ray showing multiple punched out lytic lesions

- **Renal lesions:** Renal insufficiency is one of the major cause of death which may be a result of "myeloma kidney" (light chain cast nephropathy) or hypercalcemia.
  - **Myeloma kidney**: The excretion of light chains in urine known as **Bence Jones proteinuria** is toxic to epithelial cells of renal tubules. Light-chain cast nephropathy is a characteristic finding and results from precipitation of large, waxy, laminated casts in the renal tubules. Certain light chains predispose to amyloidosis of the AL type and may result in nephrotic syndrome.
  - **Hypercalcemia:** The second most common cause of renal insufficiency is hypercalcemia, which leads to hypercalciuria and osmotic diuresis. This results in volume depletion and prerenal kidney failure. Hypercalcemia also causes deposition of calcium in the interstitium of the kidney (nephrocalcinosis).
  - **Other renal findings:** Patients are prone to **acute and chronic pyelonephritis** and sometimes deposition of uric acid crystals (urate nephropathy) may also be found.
- **Other organs**: In advanced stage, plasma cell infiltrates may be observed in the spleen, liver, lungs, lymph nodes and other soft tissues.

As the tumor load of plasma cells increases progressively in the marrow, it leads to a normocytic normochromic anemia, sometimes accompanied by moderate leukopenia and thrombocytopenia.

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Clinical Manifestations

The disease is insidious in onset. The clinical features of multiple myeloma are due to (Fig. 28.6):

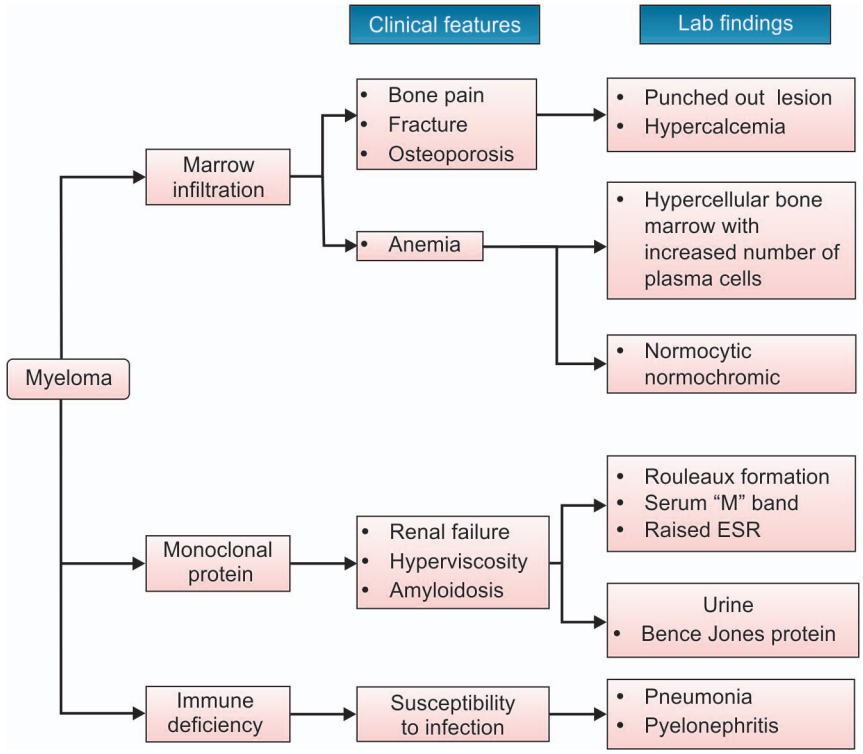


Fig. 28.6: Clinical features and lab findings in myeloma

Due to Tumor Cells Causing Bony Lesions

- **Resorption of bone**: Tumor cells along with marrow stromal cells secrete cytokines responsible for bone resorption and osteoporosis. This results in pathologic fractures and chronic bone pain and tenderness, particularly in the back or chest.
- **Compression:** Lesion in the vertebra may compress the spinal cord nerve root and cause neurological symptoms of sensory and/or motor loss.
- **Hypercalcemia:** Calcium released from the resorbed bone causes hypercalcemia and produces neurologic manifestations like confusion, weakness, lethargy, constipation and polyuria. Hypercalcemia may also cause renal damage (nephrocalcinosis).
- **Pallor:** Myeloma cells replace the normal erythropoietic foci in the bone marrow and cause progressive anemia and result in weakness and fatigue.

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Production of M Proteins (increased immunoglobulins)

- **Bleeding tendency**: Platelets may get coated by M proteins and their adhesion and aggregation is impaired resulting in bleeding.
- **Coagulation abnormalities**: M components form complexes with coagulation factors causing coagulation abnormalities.
- **Amyloidosis:** AL type amyloidosis may develop in nearly 10% of cases and may produce nephrotic syndrome, renal insufficiency or both.
- **Cryoglobulinemia and hyperviscosity:** They are uncommon. Hyperviscosity may affect the central nervous system (leading to confusion and dizziness), retina and cardiovascular system.

Humoral Immune Deficiency

- **Humoral immunity:** Humoral immune deficiency may be due to suppression of normal B lymphocytes and decreased production of normal serum immunoglobulins. This predisposes to recurrent bacterial infections, particularly pneumonia and pyelonephritis.
- **Cellular immunity:** This is relatively unaffected.

Renal Disease

Renal damage is multifactorial and is a result of Bence Jones proteinuria, hypercalcemia, humoral immune deficiency, amyloidosis and dehydration. The symptoms may be secondary to renal insufficiency, infections or nephrotic syndrome.

Prognosis

Multiple myeloma is a disease with progressive course and generally has a poor prognosis. The median survival is about 3 to 4 years. Death usually results from infection or renal failure.

Clinical Variants of Plasma Cell Myeloma

Asymptomatic (smoldering) plasma cell myeloma
In asymptomatic plasma cell myeloma, the serum M protein is at myeloma level (more than 30 gm/L) and/or 10% or more clonal plasma cells in the bone marrow, but without any related organ or tissue damage. The patients are asymptomatic and carry a higher risk of progression to myeloma or related malignancy compared to MGUS.

Nonsecretory Myeloma

This is a form of multiple myeloma in which there is no M protein in either serum or urine. Renal involvement is less common than in myeloma.

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Plasma Cell Leukemia

Definition: Plasma cell leukemia (PCL) is the term used when the peripheral blood has clonal plasma cells more than 2000/cu mm (2×10^9/L) or constitutes 20% or greater of the differential leukocyte count.

Plasma cell leukemia may be classified as primary when it is diagnosed in the leukemic phase (60%) or as secondary when leukemic transformation develops in a patient with plasma cell myeloma (40%).

Primary plasma cell leukemia develops in younger individuals and presents with hepatosplenomegaly and lymphadenopathy, a higher platelet count, few bone lesions, mild serum M protein component and longer survival rate, when compared to those with secondary plasma cell leukemia.

PLASMACYTOMA

It is characterized by localized proliferation which forms a single discrete plasma cell tumor in bone (usually) or soft tissue.

Sites: They may occur either in the bone or soft tissue.

Solitary Plasmacytoma of Bone

Definition: Solitary plasmacytoma of bone (osseous plasmacytoma) is a localized bone tumor composed of monoclonal plasma cells.

- **Bone lesions** usually involve the same sites as in multiple myeloma.
- Solitary plasmacytoma of the bone often represents an early stage and usually progresses to classic multiple myeloma.

Extra-osseous (extramedullary) Plasmacytoma

Definition: Extra-osseous plasmacytomas are localized plasma cell neoplasms that arise in tissues other than the bone.

- **Sites:** Extra-osseous lesions are usually found in the upper respiratory tract, especially in the nasal cavity and sinuses, nasopharynx and larynx.
- In contrast to osseous plasmacytoma, in extraosseous plasmacytoma dissemination occurs only in few patients.

IMMUNOGLOBULIN DEPOSITION DISEASES

Primary amyloidosis: It is caused by plasma cell neoplasm or rarely a lymphoplasmacytic neoplasm that secretes intact or fragments of abnormal immunoglobulin light chains. These light chains are deposited in various tissues and form a β-pleated sheet structure (AL amyloid).

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MONOCLONAL GAMMOPATHY OF UNCERTAIN SIGNIFICANCE

Definition: Monoclonal gammopathy of uncertain significance (MGUS) is defined as presence of serum M protein concentration lower than 3 gm/dL, bone marrow clonal plasma cells less than 10% in an asymptomatic patient.

MGUS is one of the most common plasma cell dyscrasias, occurring in about 3 to 5% of older individuals.

Etiology: MGUS may be considered as initial nonmalignant proliferation of monoclonal plasma cells. The etiology of MGUS is unknown. MGUS often has the same chromosomal abnormalities as those found in multiple myeloma, suggesting that MGUS may represent an early stage of myeloma development.

Clinical manifestations: MGUS is asymptomatic and usually diagnosed incidentally while investigating for some other disease on laboratory testing.

Prognosis: Most of the patients remain stable without any increase in M protein. However, they can progress to plasma cell myeloma. Thus, it should be considered as pre-neoplastic condition.

OSTEOSCLEROTIC MYELOMA

Osteosclerotic myeloma (POEMS syndrome) is a rare plasma cell neoplasm characterized by fibrosis and osteosclerotic changes in bone trabeculae. This disorder is often a component of a rare syndrome called POEMS. The features of POEMS includes:

P—Polyneuropathy
O—Organomegaly-lymphadenopathy, splenomegaly, hepatomegaly.
E—Endocrinopathy-hypogonadism/ hypothyroidism
M—Monoclonal gammopathy
S—Skin changes-hyperpigmentation, hypertrichosis and Raynaud's phenomenon.

SUMMARY

- Plasma cell neoplasms are group of B cell neoplasms associated with proliferation of single clone of immunoglobulin-secreting plasma cells.
- Plasma cell myeloma is characterized by the proliferation of plasma cells in the bone marrow, multiple lytic lesions in bones, monoclonal gammopathy ("M protein") and hypercalcemia. Urine may contain light chains and is known as Bence Jones proteinuria.
- Plasma cell leukemia is characterized by the presence of more than 20% plasma cells in the peripheral blood.
- If proliferation of plasma cells is localized forming single discrete tumor, it is known as solitary plasmacytoma.
- MGUS is defined as presence of a serum M protein concentration lower than 3 gm/dL in an asymptomatic patient.

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SELF-ASSESSMENT EXERCISES

I. Essay

Describe the clinical features, bone marrow findings and laboratory diagnosis of multiple myeloma.

II. Short Notes

1. Laboratory diagnosis of multiple myeloma
2. Solitary myeloma (plasmacytoma)
3. Plasma cell leukemia
4. Bence Jones protein and how to demonstrate it

III. Multiple Choice Questions

1. **The following is NOT a major pathologic feature of multiple myeloma**
   A. Hypercalcemia
   B. Polyclonal gammopathy
   C. Multiple lytic lesions in bones
   D. Proliferation of plasma cells in the bone marrow
2. **The following is NOT a criterion for the diagnosis of multiple myeloma**
   A. Lytic bone lesion
   B. Bence Jones protein
   C. Plasma cells in BM > 30%
   D. Decreased β_2 microglobulin
3. **Bence Jones proteins represent**
   A. Low molecular weight albumins
   B. Light chain immunoglobulins
   C. Heavy chain immunoglobulins
   D. High molecular weight albumins
4. **The tumor mass of multiple myeloma is formed by**
   A. Clonal expansion of marginal zone B cells
   B. Pre-T cells proliferating in the marrow
   C. Circulating B cells which proliferate in BM
   D. Post-germinal center B cells
5. **Flame cells are seen in**
   A. Multiple myeloma
   B. Acute myeloid leukemia
   C. Myeloproliferative neoplasm
   D. Myelodysplastic syndrome
6. **Multiple myeloma may show all the following features EXCEPT**
   A. Raised ESR
   B. Raised serum calcium
   C. Rouleaux formation
   D. Osteoblastic bone lesions

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7. **In multiple myeloma, cells with blue grape like cytoplasmic inclusions are known as**
   A. Mott cells
   B. Flame cells
   C. Plasmablasts
   D. Immature myeloma cells
8. **Russell bodies and Dutcher bodies are found in**
   A. Chronic lymphocytic leukemia
   B. Burkitt lymphoma
   C. Myelodysplastic syndrome
   D. Multiple myeloma
9. **"Multiple punched out osteolytic lesions" on X-ray examination is a feature of**
   A. Osteosarcoma
   B. Ewing sarcoma
   C. Chondrosarcoma
   D. Multiple myeloma
10. **In multiple myeloma, higher value of serum β_2 microglobulin is**
    A. A poor prognostic marker
    B. Required for its diagnosis
    C. Associated with renal infarction
    D. Associated with flame cells in the bone marrow

ANSWERS

1. B **2.** D **3.** B **4.** D **5.** A **6.** D **7.** A **8.** D
9. D **10.** A

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CHAPTER 29 Lymphopoietic System

CHAPTER OUTLINE

- Introduction
- Lymph Nodes
- Development of Lymphocytes

INTRODUCTION

The lymphopoietic system consists of lymphocytes and lymphoid organs.

- **Lymphocytes:** They are derived from bone marrow hematopoietic stem cells (HSCs) and are mainly of two types.
  - **T lymphocytes (T cells):** These cells differentiate and mature in the thymus.
  - **B lymphocytes (B cells):** They develop in the bone marrow.
- **Lymphoid organs:** They are divided into:
  - **Primary lymphoid organs:** It consists of bone marrow and thymus which are the primary sites of lymphopoiesis.
  - **Secondary (peripheral) lymphoid organs:** These include *lymph nodes, spleen and mucosal lymphoid tissues* [e.g. upper airways, BALT (bronchus-associated lymphoid tissue), gastrointestinal tract-MALT (mucosa-associated lymphoid tissue)]. These are the sites of T lymphocyte and B lymphocyte activation resulting in the formation of immunocompetent T and B lymphocytes.

LYMPH NODES

Size and Shape

Lymph nodes are secondary (peripheral) lymphoid organs that range from 2 to 20 mm in diameter and are discrete ovoid or bean-shaped.

Capsule

Lymph nodes are surrounded by a connective tissue capsule.

Subcapsular Sinus

Beneath the capsule is a slit-like space called the subcapsular sinus. It receives lymph or interstitial fluid from the afferent lymphatics which slowly passes through the node,

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consequently collecting in medullary sinusoids and exits through a single efferent lymphatic in the hilum.

Parenchyma

The parenchyma of normal lymph node is divided into outer cortex and inner medulla.

Cortex

Superficial cortex

- It contains spherical or egg-shaped nodules of **B lymphocytes** which form lymphoid follicles. If these "virgin" B lymphocytes are unexposed to antigen, they compose homogeneous **primary follicles.** After the B lymphocytes have been stimulated by antigenic challenge, primary follicles develop central area of round, pale-staining **germinal centers** and these follicles are now referred to as **secondary follicles.** The germinal center is the main site of B lymphocyte activation and generates immunoglobulin-secreting plasma cells in response to antigenic challenge. Activated B lymphocytes within the germinal center of lymphoid follicles are known as **follicle center cells,** which consist of centroblasts and centrocytes.
- Antigen enters the lymph node via the afferent lymphatics and is trapped on the surface of specialized **antigen-presenting dendritic reticulum cells (DRCs).** These cells have long cytoplasmic processes **(stellate) which present antigens to 'virgin' (naïve) B lymphocytes** in the presence of T-helper cells. These naïve B lymphocytes subsequently undergo a series of both morphological and functional changes (Fig. 29.1).
- The germinal centers of the **reactive lymphnodes** show distinct zonation into a dark zone and light (pale) zone. This is known as **polarization.**
  - The **dark zone** is oriented towards the center of lymphnodes (facing medulla) and is composed of predominantly **centroblasts** and **tingible body macrophages** (which phagocytose the cellular debris generated by apoptosis of B lymphocytes).
  - The **light zone** faces (oriented towards) the subcapsular sinus (facing the capsule). It is composed mainly of **centrocytes, T lymphocytes** and the greatest density of **DRCs.**
- The pale staining germinal center of a secondary follicle is surrounded by a **mantle zone** consisting of small, round, compact naïve B lymphocytes and a few T lymphocytes.
- In florid B lymphocyte reactions, a population of post-germinal center B lymphocytes may accumulate adjacent to the mantle zone; which is termed as **marginal zone** (Fig. 29.1). B lymphocytes of marginal zone are larger and have more abundant cytoplasm than mantle cells. These cells are difficult to identify in normal lymphnode but can be easily identified in the pale zone of slightly larger lymphocytes surrounding the mantle zone of splenic follicles.

Paracortex

The paracortex (also known as the deep cortex) is the **T cell dependent** region lying between primary follicles. It consists of numerous evenly dispersed small T lymphocytes with a predominance of the T-helper subset. The cluster of differentiation (CD) 4 antigen (CD4+) is expressed by these helper T cells.

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Medulla

Deep to the cortex, lies the medulla having medullary sinuses and medullary cords. They contain numerous plasma cells and are one of the main sites of antibody secretion within the lymph node. The lymph drains through medullary sinuses to the hilum.

DEVELOPMENT OF LYMPHOCYTES (LYMPHOPOIESIS)

Lymphocytes arise from the hematopoietic stem cells (HSCs) in the bone marrow. The pluripotent HSCs on commitment to development, give rise to two types of multipotent progenitor cells (refer page 6-8) namely common myeloid progenitor (CMP) and **common lymphoid progenitor (CLP).** Common lymphoid progenitor gives rise to lymphocytes.

Development of B Lymphocyte (Figs 29.1 and 29.2)

Mature B lymphocytes comprise about **15-20% of circulating lymphocytes.** The term B was originally coined for the bursa of Fabricius, the avian organ from which immunoglobulin-producing cells are derived. In humans, during adult life, B lymphocytes are generated in the bone marrow. Lymphocyte development is associated with sequential gain as well as loss of several cytoplasmic and surface antigens. The pattern of expression of these antigens identifies the character of the cells or the maturation stage of a neoplastic clone.

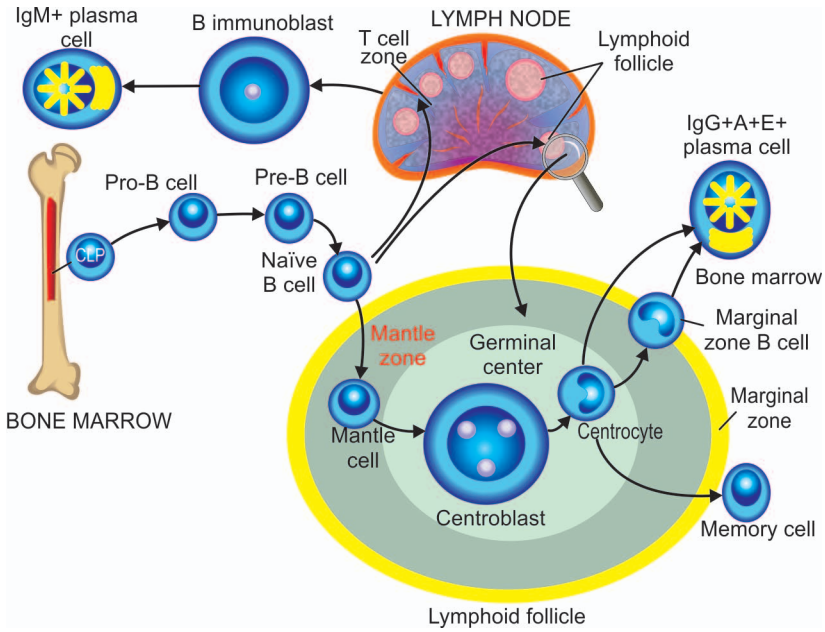


Fig. 29.1: Development of B lymphocytes. (CLP = Common lymphoid progenitor)

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Stages

Normal B lymphocyte differentiation can be divided into two stages.

First stage

- **Common lymphoid progenitor (CLP)** cell gives rise to the first recognizable B lymphocyte in humans and is known as **pro-B (progenitor) cell.** It is characterized by receptor CD19 and TdT, but without any cytoplasmic or surface bound immunoglobulin.
- Pro-B cell differentiates into the **pre-B cell** and is characterized by the presence of intracytoplasmic μ heavy chains but without any surface-bound immunoglobulin. It also contains HLA-DR antigens, CDI9, CD79a and other surface markers. These pre-B cells are **also known as B-lymphoblasts** (blast cells that are precursors of the entire B cell line).
- Pre-B cells give rise to **naïve (virgin) B cells.** These cells characteristically express immunoglobulin (antigen) receptors on their surface that have not encountered antigen. Naïve B cells (often CD5+) are small resting lymphocytes **present in primary lymphoid follicles,** in follicular **mantle zones** and in the **circulation.**
- The movement of naïve B cells from blood stream into secondary lymphoid organs and back into blood stream is known as **lymphocytic recirculation.**

Second stage

On encountering antigen, naïve B cells undergo transformation, proliferate and finally mature into antibody-secreting plasma cells and memory B cells. This occurs predominantly in the secondary (peripheral) lymphoid organs; either in the germinal center (follicular area) or outside the germinal center (extra follicular area).

Development in the germinal center (follicular area):

- Antigen exposed naïve B cells migrate into the germinal center of lymphoid follicle, proliferate and transform into large follicular center cells called **centroblasts** which express Bcl-6 protein and CD10.
- Centroblasts mature and give rise to cleaved follicular center cells known as **centrocytes** (small lymphocytes with cleaved nuclei). These centrocytes differentiate either as:
  - **Plasma cells** (IgG+A+E+): These cells produced in the germinal center, enter peripheral blood and home the bone marrow. They secrete circulating immunoglobulins (IgG+A+E+). **Plasma cells** are terminally differentiated effector B lymphocytes and are characterized by abundant cytoplasmic immunoglobulins. Plasma cells have eccentric nuclei with clumped chromatin marginated at the nuclear membrane, classically known as clock face chromatin. The cytoplasm is abundant blue-purple and often displays a clear perinuclear zone or hof representing the Golgi complex.
  - **Memory B cells:** These **post-germinal center** memory B cells are responsible for the rapid humoral response when the same antigen is encountered again. They are present in the marginal zone of the follicle.
  - **Marginal zone B cells:** They can also differentiate into plasma cells.

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Development outside the germinal center (extra follicular area):

- In the early primary immune response, the naïve B cells that encountered antigen may transform into **B immunoblasts, in the T cell zones** (Fig. 29.1). The B immunoblasts mature into IgM+ plasma cells secreting early IgM antibody as a primary immune response to antigen. The B immunoblats are large cells with abundant cytoplasm, eccentric nucleus with prominent central nucleoli and prominent Golgi zone. The corresponding tumors arising from these cells are lymphoplasmacytic lymphoma or Waldenström's macroglobulinemia.

Development of T Lymphocyte (Fig. 29.2)

Mature T lymphocytes comprise **60-80% of circulating lymphocytes.**

Stages

T lymphocyte arises from a bone marrow precursor and undergoes maturation and differentiation in the thymus. T lymphocyte maturation is a complex process and occurs in several stages. At least three stages with multiple intermediate sub stages have been defined.

First stage

Pro-T (progenitor) cells migrate from either the bone marrow or the fetal liver to the cortex of the thymus.

Second stage

Pro-T cells migrate first from the subcapsular cortex to the inner cortex and then to the thymic medulla. During this period, T cells with the ability to recognize foreign antigens are retained, while T cells with the ability to recognize self-antigens are eliminated. The double (CD4/CD8) negative pro-T cells mature into double (CD4/CD8) positive pre-T cell.

Third stage

In the thymic medulla, the **pre-T** cells lose the ability to make terminal deoxynucleotidyl-transferase (TdT), as they convert to mature T cells. These pre-T cells are **also known as T-lymphoblasts.**

- The **mature T** cells then circulate in the blood and peripheral or secondary lymphoid tissues as **post-thymic (peripheral) T cells.**
- There are two major subsets of mature T cells.
  - **T-helper (CD4+) cells** which are responsible for the cell mediated immunity, graft rejection and help the B cells in production of antibodies.
  - **T-suppressor/cytotoxic (CD8+) cells** are involved in cell-mediated cytotoxicity, apoptosis, transplant rejection and immune invasion.

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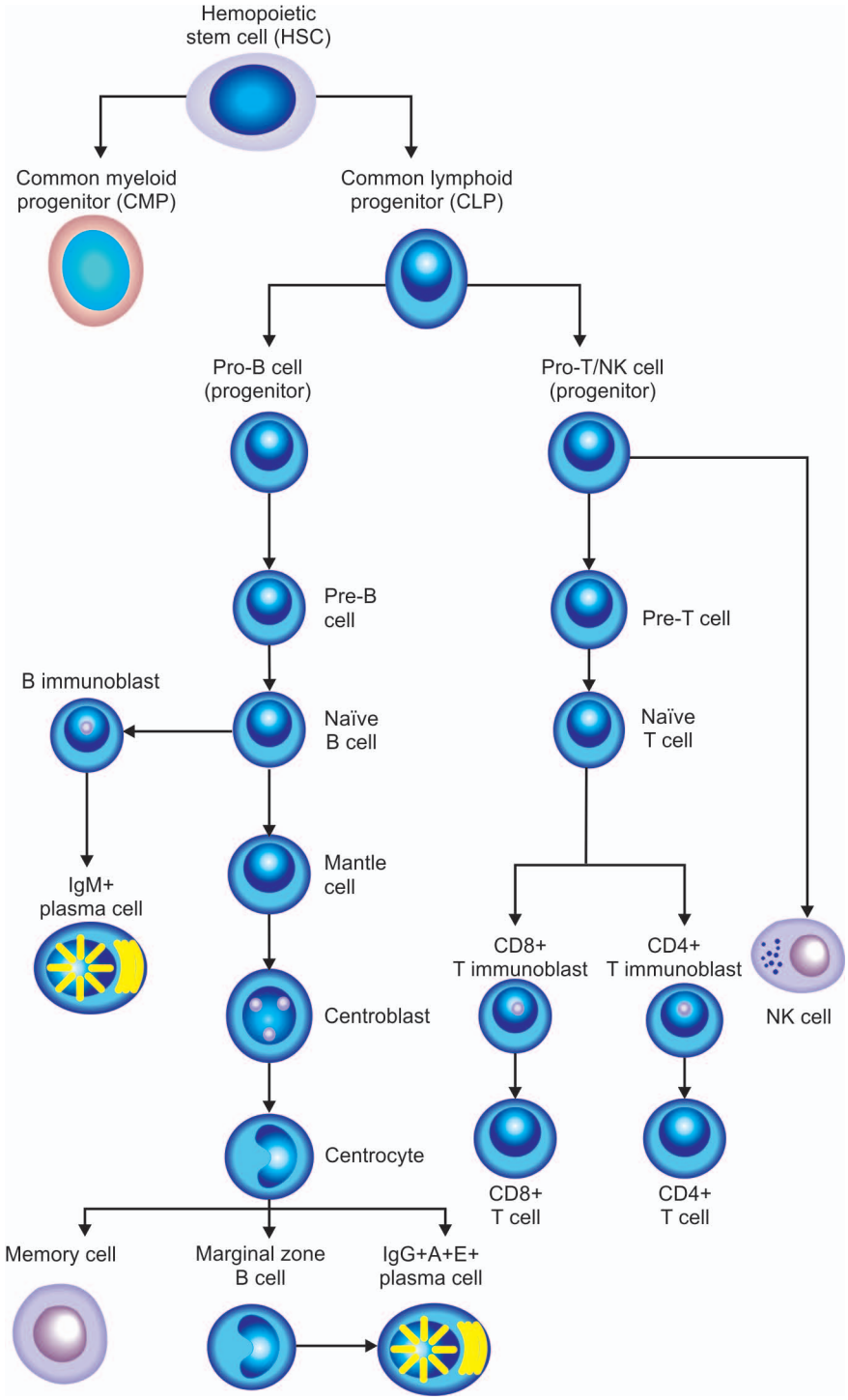


Fig. 29.2: Different stages in lymphopoiesis of both B and T cells

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Natural Killer (NK) Cell

A small proportion of lymphocytes does not express B or T cell differentiation antigens and are known as NK cells. These are large lymphocytes with granular cytoplasm (Fig. 29.3). They are capable of killing tumor and virus infected cells directly as well as through antibody mediated cytotoxicity.

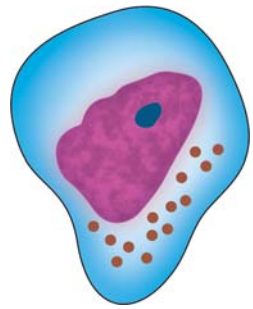


Fig. 29.3: NK cell with granular cytoplasm

SUMMARY

- Lymphopoietic system consists of lymphocytes and lymphoid organs.
- Lymphocytes are mainly of two types namely, B and T lymphocytes and are derived from the common lymphoid progenitor (CLP).
- Lymphoid organs consist of primary and secondary lymphoid organs.
  - The primary lymphoid organs being bone marrow and thymus and are the primary sites of lymphopoiesis.
  - Secondary lymphoid organs mainly include lymph nodes, spleen and mucosal lymphoid tissues.
- B lymphocytes develop from maturation of CLP into pro-B cell, pre-B cells and naïve cells which enter into the secondary lymphoid organs. In the germinal center of secondary organs like lymphnode, they transform into mantle cell, centroblasts and centrocytes. Centrocytes either mature to form immunoglobulin secreting plasma cells or memory cells. Naïve B cells can also transform into B immunoblasts in the T-cell zones, which are the precursors of IgM secreting plasma cells involved in the primary immune response.
- T lymphocytes are also derived from CLP which differentiate into pro, pre-T cells and finally mature to form either CD4+ (helper) or CD8+ (cytotoxic) lymphocytes.

SELF-ASSESSMENT EXERCISES

I. Short Notes

1. Types of lymphocytes
2. Lymphopoiesis

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II. Multiple Choice Questions

1. **In the lymph node, rim of small, round, compact B lymphocytes surrounding the germinal center is known as**
   A. Mantle zone
   B. Marginal zone
   C. Paracortical zone
   D. Postgerminal center
2. **T-suppressor cells are also known as**
   A. Cytotoxic cells
   B. CD4+
   C. NK cells
   D. Null cells

ANSWERS

1. A **2.** A

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CHAPTER 30 Lymphoid Neoplasms

CHAPTER OUTLINE

- ❖ Introduction
- ❖ Classification of Lymphoid Neoplasms
- ❖ Precursor Lymphoid Neoplasms
- ❖ Mature B cell Neoplasms
- ❖ Mature T cell And NK-cell Neoplasms

INTRODUCTION

Lymphoid neoplasms are a heterogeneous group of disorders composed of cells that resemble one or more stages of normal lymphocyte development (Figs 29.1 and 29.2).

Categorization of Lymphoid Neoplasms

The stage of lymphocyte development at which neoplastic transformation occurs, determines the site where the malignant cells first appear and their subsequent metastatic behavior.

- ***Lymphocytic/lymphoid Leukemia:*** Lymphoid neoplasms arising from **precursor** (pre-B or pre-T) **lymphoid cells**, usually present with peripheral blood and bone marrow involvement. They are known as lymphocytic leukemias. They clinically present with signs and symptoms related to **suppression of normal hematopoiesis** due to infiltration of the marrow by tumor cells. Lymphocytic leukemias also characteristically infiltrate spleen and liver resulting in their enlargement.
- ***Lymphoma:*** Lymphoid neoplasm of **mature** (B and T/NK) **lymphoid cells** usually present as tumor of lymphnodes **(lymphadenopathy)** or related organs (e.g. spleen, liver and other solid tissues). Lymphomas arising in the **lymphnodes** are known as **nodal** and those in the lymphoid related organs are known as **extranodal lymphomas** (e.g. skin, stomach or brain). Within the lymphomas, **Hodgkin lymphoma (HL)** is segregated from all other lymphoid neoplasms, which constitute **non-Hodgkin lymphoma (NHL).**

Lymphoma may spread to involve blood and marrow and leukemia may spread to lymph nodes or related organs.

CLASSIFICATION OF LYMPHOID NEOPLASMS

World Health Organization **(WHO)** classification (2008) divides the lymphoid neoplasms into **four broad categories,** based on their cell of origin (Table 30.1). This classification also considers the clinicopathological and immunological profile (Table 30.2) and has a better clinical and therapeutic importance.

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Table 30.1: WHO Classification of the lymphoid neoplasms (2008)

I. PRECURSOR LYMPHOID NEOPLASMS
B lymphoblastic leukemia/lymphoma
T lymphoblastic leukemia/lymphoma

II. MATURE B CELL NEOPLASMS
Chronic lymphocytic leukemia/small lymphocytic lymphoma
B cell prolymphocytic leukemia
Splenic B cell marginal zone lymphoma
Hairy cell leukemia
Lymphoplasmacytic lymphoma
Heavy chain disease
Plasma cell neoplasm
Follicular lymphoma
Mantle cell lymphoma
Diffuse large B cell lymphoma
Burkitt lymphoma

III. MATURE T AND NK CELL NEOPLASMS
T cell prolymphocytic leukemia
T cell large granular lymphocytic leukemia
Mycosis fungoides
Sézary syndrome
Peripheral T cell lymphoma, NOS
Angioimmunoblastic T cell lymphoma
Anaplastic large cell lymphoma
Adult T cell leukemia/lymphoma
Extranodal NK/T cell Lymphoma, nasal type

IV. HODGKIN LYMPHOMA
Classical Hodgkin lymphoma
Nodular sclerosis classical Hodgkin lymphoma
Mixed cellularity classical Hodgkin lymphoma
Lymphocyte-rich classical Hodgkin lymphoma
Lymphocyte depleted classical Hodgkin lymphoma
Nodular lymphocyte predominance Hodgkin lymphoma

Table 30.2: Cell type and its antigens detected by monoclonal antibodies

Cell type	*Antigen designation*
T cell	CD1, CD3, CD4, CD5, CD8
B cell	CD10, CD19, CD20, CD21, CD23, CD79a
Monocyte or macrophage	CD11c, CD13, CD14, CD15, CD33, CD64
NK cell	CD16, CD56
Stem cell and progenitor cell	CD34
All leukocytes	CD45(LCA)

CD, cluster designation; NK, natural killer; LCA, leukocyte common antigen

General Features of Lymphoid Neoplasms

- **Majority** (80 to 85%) of the lymphoid neoplasms are of **B cell origin** and remaining of T cell/NK cell type. Most of the lymphoid neoplasms resemble some stage of B or T cell differentiation.

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- Lymphoid neoplasms **disrupt the immune system** and these patients are susceptible to autoimmune diseases or infections. Patients with immunodeficiency (inherited or acquired) are at high risk of developing certain lymphoid neoplasms.
- Neoplastic (B and T) cells usually **recapitulate the structure and behavior of their normal counterparts.** For example, follicular lymphomas of the lymph node produce a nodular or follicular pattern of growth, whereas T cell lymphomas grow in paracortical T cell zones.

PRECURSOR LYMPHOID NEOPLASMS

Acute lymphoblastic leukemia/lymphoma is discussed in Chapter 22.

MATURE B CELL NEOPLASMS

Chronic lymphocytic leukemia (CLL)/small lymphocytic lymphoma (SLL), prolymphocytic leukemia and hairy cell leukemia are discussed in Chapter 27.

Lymphoplasmacytic Lymphoma

Definition

Lymphoplasmacytic lymphoma (LPL) is a **neoplasm of B lymphocytes** in which the tumor cells show **differentiation towards plasma cells.** Significant subset of patients with LPL often secrete monoclonal IgM (not required for diagnosis).

Waldenström macroglobulinemia (WM) is found in significant number of patients with lymphoplasmacytic lymphoma. WM is defined as **LPL with bone marrow involvement** and **IgM monoclonal gammopathy.** In contrast to multiple myeloma, there is usually balanced synthesis of both heavy and light chains and complications due to free light chains like renal failure and amyloidosis do not develop.

Cell of Origin

Post-follicular B lymphocyte which differentiates to plasma cell.

Morphology

Sites involved
Most tumors involve the **bone marrow** and some lymphnodes and extranodal sites (spleen).

Bone marrow
- Bone marrow involvement is very common and shows a nodular, diffuse and/or interstitial infiltration composed of **small lymphocytes** admixed with **plasma cells** and **plasmacytoid lymphocytes.** The plasmacytoid lymphocytes are large having an eccentric nucleus with

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coarse chromatin. They have moderate amount of cytoplasm which is pale and basophilic. Bony erosions are absent.
- Neoplastic cells may show nuclear pseudo inclusions **(Dutcher bodies)** or cytoplasmic PAS positive globules **(Russell bodies)** of Ig.

Lymph nodes

Neoplastic cells totally or partially replace the lymph node and are morphologically similar to those observed in the bone marrow.

Spread

The tumor spreads to **spleen** and **liver** at the time of diagnosis. As the disease progresses tumor may infiltrate **nerve roots, meninges** and the **brain.**

Immunophenotype

The lymphocytic component **expresses B cell markers** like **CD20** and is usually **negative for CD5 and CD10,** whereas the plasma cell component expresses a monoclonal immunoglobulin.

Molecular Genetics

The most common cytogenetic defect is a deletion involving chromosome 6q.

Clinical Features

- It is usually observed in the **sixth or seventh decade.**
- The presenting features are usually **nonspecific** like weakness, fatigue (related to anemia) and weight loss.
- They may develop **lymphadenopathy, hepatomegaly and splenomegaly.** Anemia may develop due to marrow infiltration and autoimmune hemolysis. The hemolysis is caused by cold agglutinins, IgM antibodies that bind to erythrocytes at temperatures below 37°C.

Hyperviscosity syndrome: IgM secreted by the tumor cells causes **increased viscosity** of the blood, giving rise to hyperviscosity syndrome. The characteristic symptoms are:
- Reduced visual acuity.
- Ischemic manifestations in the central nervous system.
- Bleeding related to interaction of macroglobulins and clotting factors or platelets.
- Cryoglobulinemia (precipitation of macroglobulins at low temperatures), producing symptoms such as Raynaud's phenomenon and cold urticaria.

Prognosis

It is an **incurable** and **progressive** disease with a median survival of about 4 years. Uncommonly, it can transform into large cell lymphoma.

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Marginal Zone Lymphoma

Definition

Lymphoma composed of marginal (parafollicular) zone B lymphocytes, which represent postgerminal center memory B cells.

Cell of Origin

Postgerminal center, marginal zone B lymphocyte.

Morphology

Sites involved

It constitutes a heterogeneous group of tumors which arise within the lymph nodes **(nodal),** spleen or **extranodal** lymphoid tissues.

Lymphnode

- In the early stages, it involves only the marginal zone/interfollicular region. Residual follicles may be observed in early stages. With extensive involvement nodal architecture is effaced.
- The tumor cells can be monocytoid, centrocyte-like, or resemble small round lymphocytes.

Extranodal Marginal Zone Lymphomas of Mucosa-Associated Lymphoid Tissue (MALT Lymphoma)

It is composed of morphologically heterogeneous small B cells.

Etiology: These tumors have unusual pathogenesis and have different characteristics.

Precursor lesions: They are found in tissues affected by chronic inflammatory disorders of either infectious or autoimmune etiology. Example of infectious organism is MALT (mucosa-associated lymphoid tissues) lymphoma following **Helicobacter gastritis (stomach).** Autoimmune based chronic inflammation resulting in MALT lymphoma is seen in **Sjögren disease (salivary gland)** and **Hashimoto thyroiditis (thyroid gland).**

The **characteristic features** of these tumors are:

- They **remain localized** to the involved site for long time and spread in late stages.
- **If the causative agent is eradicated,** these tumors **may regress.** This is true with MALT lymphoma of stomach which develops due to Helicobacter pylori and this tumor may regress following antibiotic therapy against *H.pylori*.

The above features suggest that extranodal marginal zone lymphomas **start as a reactive, polyclonal** immune reaction. With chromosomal mutations and aberrations, it is **transformed** into a **monoclonal B cell neoplasm.** Then it spreads to distant sites and finally may be transformed into diffuse large B cell lymphoma.

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Follicular Lymphoma

Definition

Follicular lymphomas are tumors **composed of follicle center** (germinal center) **B cells** of lymphoid follicles (centrocytes and centroblasts). It usually has at least a partially follicular pattern.

Cell of Origin

Germinal center B lymphocyte.

Morphology

Sites involved

It usually involves **lymph nodes**, but also spleen and bone marrow.

Gross

The involved lymph nodes are usually enlarged and have smooth outer surface. Cut surface is homogeneous gray/white.

Microscopy

Pattern: The tumor cells in most cases have a purely **follicular** (nodular) growth pattern. The **architecture** of the involved **lymph node** is **replaced by** closely packed neoplastic **follicles (nodules)** of fairly uniform size that involve the cortex and medulla. These tumors may also have a **mixed pattern with follicular and diffuse** areas. The neoplastic cells may frequently infiltrate the perinodal adipose tissue (Fig. 30.1A).

At **low magnification**, follicular lymphoma resembles reactive follicular hyperplasia. But in contrast to benign reactive follicles, the **neoplastic follicles** are **poorly defined** and do **not show a mantle zone**.

Neoplastic cells: The neoplastic follicles (Fig. 30.1B), similar to normal follicles contain varying proportions of randomly distributed mixture of **two types of B cells:**

- Small cells known as **centrocytes** (small cleaved cells) and are found in most of the lymphomas. They have irregular, indented or cleaved nuclei with inconspicuous nucleoli. The cytoplasm is pale and scanty.
- Large non-cleaved cells referred to as **centroblasts**. They have round or oval nuclei with open nuclear (vesicular) chromatin, multiple (1 to 3), peripherally (marginally) located nucleoli and moderate amount of basophilic cytoplasm. They are usually 3 times the size of lymphocyte.

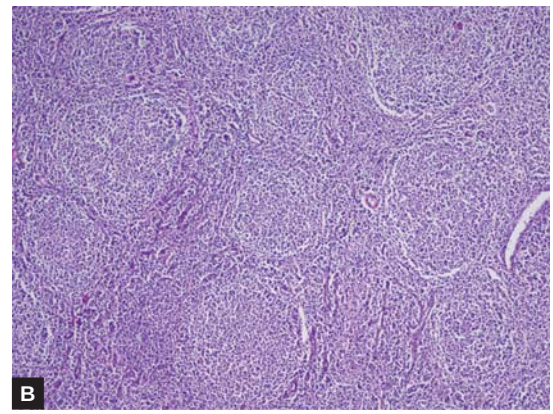
Grading

The relative proportion of centrocytes and centroblasts are of prognostic importance. Depending on the number of centroblasts they are **graded** ranging from **1 to 3**. Grade 1 with few (less than 5 centroblasts/hpf) and grade 3 with numerous centroblasts (more than 15/hpf). Higher number of centroblasts (grade 3) is associated with more aggressive course and worse prognosis.

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Figs 30.1A and B: Follicular lymphoma: (A) Neoplastic follicles in both the cortex and medulla and infiltration of the perinodal tissue: (B) Nodular aggregates of malignant lymphoid cells

Follicular lymphoma VS. follicular hyperplasia

Differentiating features are presented in Table 30.3.

Table 30.3: Differences between follicular lymphoma and reactive follicular hyperplasia

	Follicular lymphoma	*Reactive follicular hyperplasia*
Lymphoid follicles size and shape	Round and isolated	Vary greatly in size and shape
Cell polarization	Absent	Present
Mantle zone	Frequently lacking	Preserved
Nodules	Both cortex and medulla are usually involved	Only in cortex
Cells	Monotonous, monoclonal	Mixture of cells
BCL2 protein	Positive	Negative

Peripheral blood smear

May show lymphocytosis [less than 20×10^9/L (20,000/cu mm)] in approximately 10% of cases.

Involvement of bone marrow, spleen and liver is common in follicular lymphoma.

Bone marrow

It is **involved in** about **85%** of patients and is characterized by localized collections of irregular small lymphoid cells adjacent to bone trabeculae **(termed paratrabecular infiltration)**.

Immunophenotype

- It is a tumor derived from follicular center cells and expresses **CD19, CD20** (pan-B cell markers), **CD10** (CALLA) and **surface immunoglobulin.**
- Follicular lymphoma cells also express **BCL2 protein**, in contrast to normal benign germinal follicular center B cells, which are BCL2 negative.

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Cytogenetics and Molecular Genetics

- The characteristic feature of typical follicular lymphoma is a **t(14; 18)(q32:q21),** with IgH and BCL2 as partner genes and leads to constitutive **overexpression of BCL2** protein.
- **BCL2 is an anti-apoptotic gene** which prevents/inhibits apoptosis and promotes the survival of follicular lymphoma cells. Reactive follicles of lymph node normally contain numerous B cells undergoing apoptosis, whereas **neoplastic follicles** are characteristically **devoid of apoptotic cells**.

Clinical Features

- Follicular lymphoma is one of the **commonest** types of non-Hodgkin lymphoma and shows a peak age incidence in the **sixth and seventh decades**. Both males and females are equally affected.
- It presents as painless, slowly progressive, **generalized lymphadenopathy**.

Prognosis

- It is an **indolent** disease with a waxing and waning course, but it is generally regarded as incurable.
- The overall median survival is 7 to 9 years.
- The process by which follicular lymphoma is converted into a more aggressive diffuse large-cell lymphoma is called *transformation*. About 30% of cases ***transform*** into diffuse large B cell lymphoma.

Mantle Cell Lymphoma

Definition

Mantle cell lymphoma is a clinically **aggressive** B cell lymphoma which is derived from or differentiates towards the inner mantle zone B cells (which surround the germinal center).

Cell of Origin

Peripheral B lymphocyte of inner mantle zone, mostly of naïve pre-germinal center type.

Morphology

Sites involved

Lymph nodes are the most common site. Other sites include spleen, bone marrow, with or without peripheral blood involvement.

Lymphnode

- Mantle cell lymphomas may have a nodular or diffuse architecture. Lymphoid cells may be seen **surrounding reactive germinal centers** and produce a **vaguely nodular** appearance.

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- It shows homogeneous (monomorphic) population of lymphoid cells. The neoplastic cells are **small to medium-sized lymphoid cells with irregular nuclear contour and inconspicuous nucleoli.** These cells closely resemble centrocytes.
- Centroblasts (found in follicular lymphoma) and proliferation centers (observed in chronic lymphocytic leukemia/small lymphocytic lymphoma) are not seen.

Immunophenotype

- Mantle cell lymphoma **expresses CD19, CD20**, moderately high levels of surface immunoglobulin heavy chain (usually IgM and IgD) and either κ or λ light chain.
- They are usually **CD5 positive and CD23 negative**, which can help to distinguish it from chronic lymphocytic leukemia/small lymphocytic lymphoma.They are negative for CD10 and BCL6.
- It is also characteristically **positive for cyclin D1 protein**. The rearranged IgH segments typically lack somatic mutations, supporting an origin from a naïve B cell.

Cytogenetic Abnormalities and Molecular Genetics

Mantle cell lymphoma is associated with a distinctive translocation **t(11; 14)(q13;q32).** This leads to increased expression **(overexpression)** of **cyclin D1**, which promotes G_1 to S phase progression during the cell cycle.

Clinical Features

- It is usually found in the **sixth to seventh decades** of life and shows a **male** predominance.
- Common presentation is **painless lymphadenopathy**.
- **Extranodal:** It may involve bone marrow, spleen and liver. Involvement of gastrointestinal tract with a distinctive multifocal mucosal involvement is termed multiple *lymphomatous polyposis* and is associated with GIT symptoms.

Prognosis

Poor with a median survival rate of 3 to 4 years.

Diffuse Large B Cell Lymphoma (DLBCL)

Definition

Diffuse large B cell lymphoma is a **heterogeneous group** of **aggressive**, neoplasm of large B cell that has a **diffuse** growth pattern.

It constitutes about 20 to 30% of all NHL and 60 to 70% of aggressive lymphoid neoplasms.

Cell of Origin

Either germinal center or postgerminal center B lymphocyte.

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Morphology

Sites involved

DLBCL may involve **lymph nodes or extranodal** sites (most commonly in the GI tract).

Gross

The involved lymph nodes are enlarged, soft and fleshy.

Microscopy

- **Pattern:** Loss of lymph node architecture with **diffuse growth pattern** (Fig. 30.2).
- **Neoplastic cells:**
  Size: Large cell size (**4 to 5 times** the diameter of a **small lymphocyte**).
  Shape*:* Cells are round or oval.
  Cytoplasm: Moderate to abundant cytoplasm which may be pale or basophilic.
  Nucleus: The **nuclear size** equals or exceeds the **nucleus of a macrophage** or more than twice the size of a normal lymphocyte. Nuclei may have number of different appearances (Table 30.4).

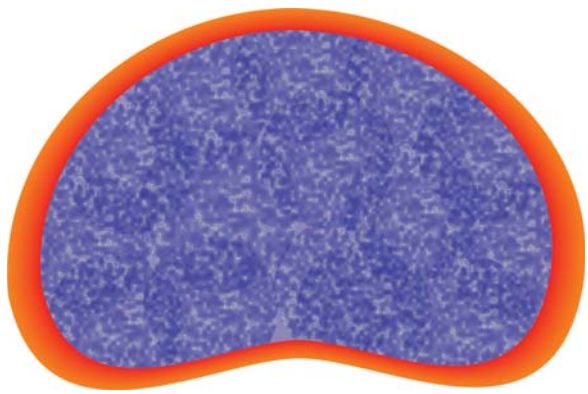


Fig. 30.2: Diagrammatic representation of diffuse lymphoma showing diffuse growth pattern

Table 30.4: Nuclear features of variants of DLBCL

Variants of DLBCL	*Nuclear features*
Common variants of DLBCL, NOS	
Centroblastic	Large vesicular nuclei and 2 to 4 peripherally (nuclear membrane-bound) arranged nucleoli
Immunoblatsic	Large vesicular nuclei with single centrally placed prominent nucleolus
Anaplastic	Large, bizarre, pleomorphic nuclei (may contain multinucleated cells that may partly resemble Reed-Sternberg cells)
Others	
T cell/histiocyte-rich	Non-neoplastic T cells/or histiocytes. Tumor cells can resemble any other variant mentioned above.
Plasmablastic	Resemble immunoblastic variant, but CD45 –ve and CD20–ve.

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Variants of DLBCL: There are three common variants and several other variants of DLBCL. The nuclear features helpful in distinguishing them are shown in Table 30.4.

Immunophenotype

The tumor cells express pan-B cell markers such as **CD19, CD20, CD22 and CD79a**. They also express germinal center markers like CD10 and BCL6. Majority may express surface Ig and all the variants are **negative for TdT.**

Cytogenetics and Molecular Profile

The commonest cytogenetic abnormalities in DLBCL are:

- **Translocation of BCL2 gene:** The t(14;18) translocation which is characteristic of follicular lymphoma may also occur in DLBCL. This results in deregulation of the anti-apoptotic BCL2 gene, which prevents apoptosis and promotes the survival of tumor cells.
- **Mutations of the BCL6 gene:** BCL6 expression is required for the formation of normal germinal centers. BCL6 can suppress p53 ("guardian of the genome"). The translocations involving the 3q27 breakpoint with overexpression of BCL gene is observed in about 30% of patients. This may prevent the activation of DNA repair mechanisms in germinal center B cells. Abnormalities of the BCL6 gene have been associated with extranodal presentations.

Etiology

The cause of DLBCL is unknown.

Special subtype associated with oncogenic viruses

- **EBV positive diffuse large B cell lymphoma of the elderly:** Occurs in elderly patients and the neoplastic B cells are usually infected with Epstein-Barr virus, which plays a role in pathogenesis.

Clinical Features

- It is more common between **65 and 70 years** of age with slight **male** preponderance.
- Usually presents with a **rapidly enlarging**, often symptomatic, mass at a single or multiple **nodal or extranodal sites.**
- Extranodal disease can arise within the gastrointestinal tract, skin, bone, brain and other sites. Bone marrow involvement usually occurs late in the disease.

Prognosis

DLBCLs are **aggressive** tumors which are **rapidly fatal** if untreated.

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Burkitt Lymphoma

Definition

Burkitt lymphoma (BL) is a distinctive type of **highly aggressive** neoplasm, often presents as **extranodal** B cell lymphoma or as an acute leukemia. It is composed of **medium-sized,** highly proliferating **monomorphic lymphoid cells** with basophilic **vacuolated cytoplasm**.

Cell of Origin

Germinal center or postgerminal center B lymphocyte.

Clinical Variants

Three variants of Burkitt lymphoma are recognized:

- **Endemic (African) BL:** This variant occurs in Africa, affects children and adolescents. It is associated with Epstein-Barr virus infection and malaria. It usually involves the **jaw** and presents as a mandibular mass. It also involves gastrointestinal tract, abdominal viscera, particularly the kidneys, adrenal glands and gonads (ovaries). Involvement of the bone marrow and peripheral blood is not common.
- **Sporadic (nonendemic) BL:** It usually presents as an **abdominal mass**. It frequently involves **ileocecum** and **peritoneum**. They are observed predominantly in **children or young adults**.
- **Immunodeficiency-associated (HIV) BL:** Lymph nodes and bone marrow are frequently involved.

Burkitt lymphomas, irrespective of the categories, are **histologically similar** and sporadic and immunodeficiency-associated Burkitt lymphomas are phenotypically also identical.

Morphology

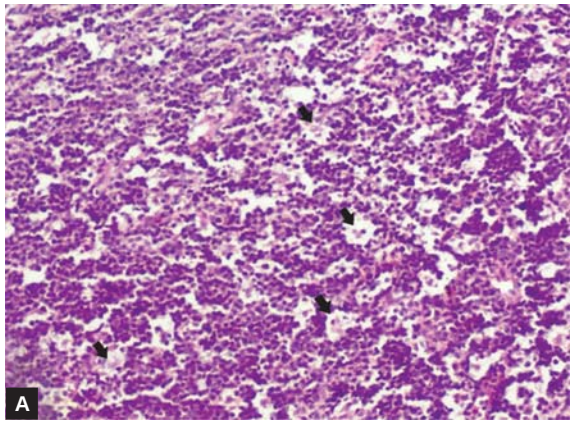
Sites involved
Most of the tumors develop at **extranodal sites** with some variation according to clinical variant. They involve the gastrointestinal tract and oropharynx.

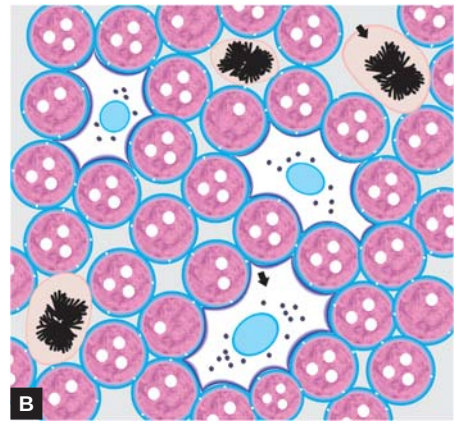
Gross
Involved nodal or extranodal regions are fleshy and may show areas of hemorrhage and necrosis.

Microscopy
The histological appearances are distinctive and the involved tissues are effaced by a **diffuse** infiltrate of **monotonous medium-sized lymphoid cells** (Figs 30.3A and B). The involved lymph node shows **loss of architecture.** The characteristic features of neoplastic cells are:

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Figs 30.3 A and B: Burkitt lymphoma composed of medium-sized lymphoid cells admixed with benign macrophages (narrow arrow) giving a "starry sky" appearance. Numerous mitotic Figures are also seen (thick arrow in B)

- **Cell size: Medium-sized** and range from 10 to 25 μm in diameter.
- **Nuclei: Round or oval nuclei** having clumped **coarse chromatin** with **several (2 to 5) nucleoli.** The nuclear size is approximately the size of the nuclei of that of benign macrophages/histiocyte.
- **Cytoplasm: Moderate** amount of deeply **basophilic** (royal blue) **cytoplasm** which tends to square off with adjacent cells. The cytoplasm shows **multiple, small**, **round lipoid** (clear) **vacuoles** in the cytoplasm. These vacuoles stain positive with oil red O.
- **High mitotic index: Mitoses** are **numerous**, with almost 100% of cells being in cell cycle.
- **Starry sky pattern:** There are also plenty of tumor cells undergoing **apoptosis.** The nuclear remnants of these apoptotic cells are phagocytosed and cleared by benign macrophages. These macrophages have abundant clear cytoplasm. They are evenly and diffusely distributed among the tightly packed sheets of basophilic tumor cells, creating a characteristic **"starry sky" pattern** (resembling stars in the sky).

Bone marrow

When involved, the aspirates show tumor cells morphologically similar to those seen in lymph node.

Immunophenotype

Burkitt tumor cells are mature B cells and express surface **IgM, monotypic κ or λ light chain** and are **positive for common B cell antigens** (CD19, CD20, and CD22). They are **positive for CD10 and BCL6** and are of germinal center origin. Burkitt lymphoma usually does not express the anti-apoptotic protein BCL2.

Cytogenetic and Molecular Genetic Features (Fig. 30.4)

Burkitt lymphoma is associated with a specific chromosomal translocation involving ***MYC* oncogene** and **EBV infection** is associated with the endemic type.

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- MYC (c-MYC) is a proto-oncogene which codes for a transcription factor and is located on chromosome 8. At its normal locus (location), its product MYC protein is tightly controlled (regulated) and expressed in all actively dividing normal cells.
- All categories of Burkitt lymphoma are associated with **translocations of the *c-MYC* gene** on chromosome 8, which results in conversion of proto-oncogene into MYC oncogene. The most common form of translocation results in the movement of the *MYC*-containing segment of chromosome 8 to chromosome 14q32, placing it close to the ***IGH* (H = heavy chain) gene** (Fig. 30.4). The genetic notation for the translocation is t(8:14) (q24;q32).

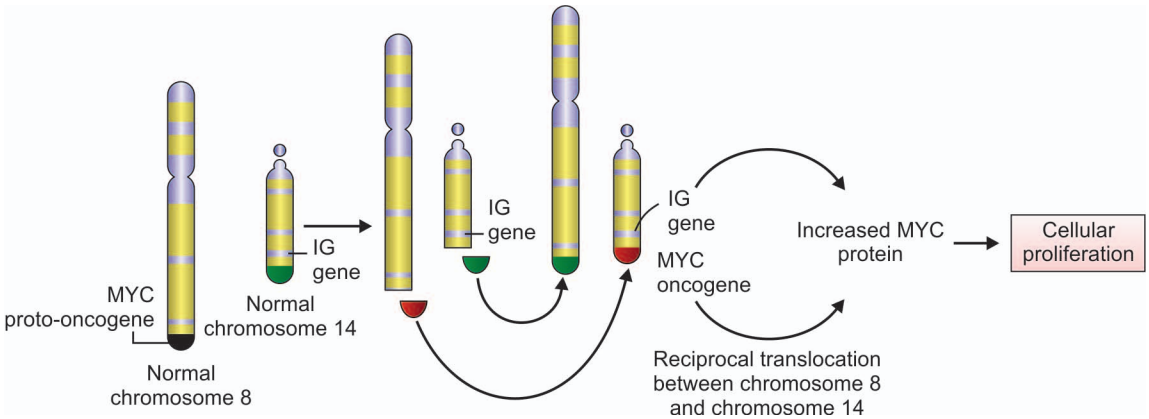


Fig. 30.4: Chromosomal translocation and activated MYC oncogene in Burkitt lymphoma

- Due to this translocation, MYC oncogene causes **persistent expression or overexpression of MYC protein** (oncoprotein). This **results in** change of regulated cell proliferation to an **uncontrolled cell proliferation** as well as stimulation of apoptosis. Apoptosis is probably necessary to allow proliferation of cells. Burkitt lymphomas also have **mutations which inactivate p53**.

All endemic tumors have latent infection with EBV, which is also present in about 20 to 25% of HIV-associated tumors and sporadic cases. Molecular analysis has shown that the configuration of the episomal EBV DNA is identical in all tumor cells within individual cases, indicating that infection precedes cellular transformation.

Prognosis

Burkitt lymphoma is **very aggressive but responds well** to short-term, high-dose **chemotherapy**.

Poor **prognostic factors are:**

- Involvement of blood, bone marrow and central nervous system.
- Bulk of the disease-unresected tumor of more than 10 cm in diameter.

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- High serum LDH levels
- Presence of residual disease after excision

Plasma Cell Neoplasms are discussed in Chapter 28.

Composite Lymphoma

Definition: When two or more histologically distinct types of lymphoma involve the **same anatomic site**, it is referred to as composite lymphoma.

MATURE T CELL AND NK CELL NEOPLASMS

These neoplasms consist of cells which phenotypically resemble mature T cells or natural killer (NK) cells. Peripheral T cell tumors **constitute less than 15% of NHLs** and NK cell tumors are very rare.

Peripheral T Cell Lymphoma, NOS

Definition

Peripheral T cell lymphoma (PTCL), NOS constitutes morphologically different subtypes of nodal and extranodal mature T cell lymphomas.

Cell of Origin

Activated mature T lymphocytes

Morphology

Sites involved
Mainly involves lymphnode, but can involve any site.

Microscopy
- There is no pathognomonic morphological feature of peripheral T cell lymphomas. The involved **lymphnode** shows **effacement of the normal architecture** due to **paracortical or diffuse infiltration** by neoplastic T cells.
- **Neoplastic T cells:**
  Size: It ranges from **small, intermediate to large**.
  Cytoplasm: It may be sparse or abundant; clear, eosinophilic or basophilic.
  Mitotic figures: Many mitotic figures may be seen. Occasionally **Reed-Sternberg-like cells** can be found.
  Nuclei: Tumor cells have vesicular or hyperchromatic nuclei, prominent nuclei and show nuclear pleomorphism.
- **Non-neoplastic reactive cells:** There are frequently inflammatory cells in the background consisting of eosinophils, plasma cells and macrophages, probably attracted by cytokines secreted by T cells.

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Immunophenotype

The diagnosis can be confirmed only by immunophenotyping. All peripheral T cell lymphomas have a **mature T cell** phenotype, **lacking TdT** (expressed by immature T cells). They usually **express pan-T cell** and T cell associated antigens including CD2, C3 ,CD5 and either αβ or γδ T cell receptors (TCR).

Clinical Features

- Age group of presentation ranges from fifth to seventh decade.
- Most present with generalized lymphadenopathy.
- Patients can present with cytokine induced paraneoplastic features such as eosinophilia, pruritus and hemophagocytic syndromes.

Prognosis

These tumors are **highly aggressive** with a poor response to therapy. These lymphomas have a significantly worse prognosis compared to mature B cell neoplasms (e.g. diffuse large B cell lymphoma).

Anaplastic Large Cell Lymphoma

Definition

Anaplastic large cell lymphoma (ALCL) is an **uncommon, high-grade** NHL of T-cell lineage but may fail to express lineage-associated markers.

Cell of Origin

Activated mature cytotoxic T lymphocytes

Morphology

Sites involved
Involves both lymph nodes (nodal) and extranodal sites (skin, bone, soft tissue).

Microscopy

- **Loss of architecture**: The lymph node shows loss of architecture with diffuse infiltration by lymphoma cells.
- **Neoplastic cells:** The neoplastic cells are **large, anaplastic** and tend to be cohesive, forming clusters and sheets. The cytoplasm is abundant. The nuclei are round, lobulated or have bizarre shapes.
  Hallmark cells: These characteristic cells typically contain **horseshoe (pig-embryo/kidney) shaped eccentric nuclei,** often with an eosinophilic region near the nucleus. These cells are referred as the 'hallmark' cells because they are **present in all ALCLs.**

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Immunophenotype

Anaplastic lymphoma kinase (ALK) is not expressed in normal lymphocytes or other lymphomas. Detection of **ALK protein** in tumor cells is a reliable indicator of an ALK gene rearrangement.

Cytogenetic and Molecular Genetic Features

ALCL has a strong association with chromosomal translocation, **t(2;5)(p23;q35)**, which leads to the aberrant **expression of a chimeric (fusion) protein** containing a tyrosine kinase called ***anaplastic lymphoma kinase*** **(ALK)**. Among the lymphoid neoplasms, ALK rearrangements are **specific for this entity**. ALK fusion proteins constitutively activate tyrosine kinases which trigger numerous signaling pathways, including the JAK/STAT pathway.

Clinical Features

They are usually seen in **children or young adults** and frequently **involve soft tissues**.

Prognosis

Despite its aggressive histological appearance, ALCL has a relatively good overall survival rate.

Adult T Cell Leukemia/Lymphoma (ATLL)

Definition

Adult T cell leukemia/lymphoma is a neoplasm of CD4+ T cells and develops in adults infected by **human T cell leukemia virus type 1 (HTLV-1).** The clonal HTLV-1 provirus is found in the tumor cells.

Morphology

Sites involved
It shows widespread involvement of **lymph nodes** and **peripheral blood**.

Microscopy
Neoplastic cells with **multilobated nuclei** and petal-like nuclear lobules connected by thin chromatin strands, known as **"cloverleaf**" or "**flower"** cells are found in the involved tissues and peripheral blood. Less commonly, **Reed-Sternberg like cells** may also be **Anaplastic** present.

Clinical Features

It presents with generalized lymphadenopathy and ATLL cells in peripheral blood. It is often associated with skin lesions and hypercalcemia.

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Prognosis

Most of the cases progress rapidly and are fatal.

Mycosis Fungoides

Definition

Mycosis fungoides is a type of **cutaneous T cell lymphoma** composed of small to medium-sized **lymphoid cells with irregular nuclear outlines**. The tumor cells show predilection for the epidermis (**epidermotropism**).

Age

Most of the patients are adults or elderly.

Morphology

Site involved
As a rule these lymphomas are **limited to skin**.

Stages

The degree of cutaneous infiltrate is related to the stage of the disease. There are three stages:
Patch stage (inflammatory premycotic phase), characterized by erythematous macules usually occurring on areas not exposed to sunlight.
Plaque stage, with elevated scaly plaques which may be pink or red/brown and are often intensely pruritic.
Tumor stage, with dome-shaped firm tumors which may ulcerate.

Microscopy

- The **epidermis and upper dermis** is infiltrated by small-to-medium-sized neoplastic T cells. These cells have highly **convoluted (cerebriform) nuclear contours**, due to marked infolding of the nuclear membrane. With progression of tumor the tumor cells increase in number and size.
- In some cases, intraepidermal nests of tumor cells (**Pautrier microabscesses**) may be seen. As the disease progresses, it spreads to the extracutaneous sites like lymph nodes and bone marrow.

Immunophenotype

Tumor cells express pan-T cell markers CD3, CD5 and CD2.

Sézary Syndrome

Definition

Sézary syndrome is rare disease and is defined by the triad namely:

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- **Widespread exfoliative erythroderma:** Although skin involvement is the characteristic clinical presentation, the tumor cells are found in early stage of the disease in blood, bone marrow and lymph nodes. The basis for the striking epidermotropism is uncertain.
- **Generalized lymphadenopathy**
- **Presence of characteristic Sézary cells:** They are seen in the skin, lymph nodes and peripheral blood. Sézary cells are **neoplastic T cells** with cerebriform nuclei.

Morphology

Sites involved

Sézary syndrome is a leukemia and thus is a generalized disease.

Microscopy

It may be similar to mycosis fungoides. However the cellular infiltrate is more often monotonus.

- Cellular infiltrates in Sézary syndrome are **monotonous** and epidermotropism may sometimes be absent.
- In contrast to mycosis fungoides, the skin lesions rarely proceed to tumor stage.

Immunophenotype

Tumor Cells are CD2+, CD3+ and CD5+.

Prognosis

This is an aggressive disease and most patients die of opportunistic infections.

Angioimmunoblastic T cell Lymphoma

Definition

Angioimmunoblastic T cell lymphoma (AITL) is a rare type of peripheral T cell lymphoma. It is characterized by systemic disease, widespread lymphadenopathy, skin rashes, polyclonal hypergammaglobulinemia and evidence of immunodeficiency.

Cell of Origin

CD4+ helper T lymphocytes

Morphology

Three common features seen in the involved lymph node are:

- **Diffuse pattern** with partial effacement of architecture.
- Involvement of **paracortical region** with **polymorphous infiltrate** of neoplastic T cells.
- **Prominent** proliferation of **high endothelial venules (HEV**) and follicular dendritic cells.

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Immunophenotype

Tumor Cells are CD2+, CD3+ and CD5+.

Prognosis

Angioimmunoblastic T cell lymphoma is an aggressive tumor with poor prognosis.

SUMMARY

- Lymphomas are malignant neoplasms of lymphoid system arising at specific stages of differentiation of normal lymphoid cells.
- WHO classifies the lymphoid neoplasms into four broad categories based on their cell of origin as: precursor lymphoid neoplasm, mature B or T lymphoid neoplasm and Hodgkin lymphoma.
- Majority (80 to 85%) of lymphoid neoplasms are of B-cell origin and remaining of T cell.
- Follicular lymphomas are indolent neoplasms derived from germinal center B cells. They form neoplastic follicles which contain a mixture of mainly two types of cells in varying proportions namely, centrocytes and centroblasts. They usually present as generalized lymphadenopathy in older adults with marrow involvement.
- Diffuse large B cell lymphoma is an aggressive neoplasm composed of heterogeneous group of large cells. They are most commonly seen in adults as a single rapidly growing mass.
- Burkitt lymphoma is a highly aggressive B cell lymphoma seen in adolescents or young adults either in the jaw or as an extranodal abdominal mass. They are composed of medium-sized, highly proliferating lymphoid cells with basophilic vacuolated cytoplasm. These have a "starry sky" appearance on light microscopy. Burkitt lymphomas are associated with translocations of the MYC gene on chromosome 8. African (endemic) types usually have latent infection with EBV.
- Peripheral T Cell and NK Cell neoplasms constitute less than 15% of NHLs.
- Mycosis fungoides and Sézary syndrome are T cell neoplasm which involves skin.

SELF-ASSESSMENT EXERCISES

I. Essays

1. Classify non-Hodgkin lymphoma and describe the morphological features of Burkitt lymphoma
2. Classify lymphoid neoplasms and describe the morphological features of follicular lymphoma

II. Short Notes

1. Non-Hodgkin lymphoma
2. Name peripheral B cell lymphoma
3. Differences between follicular lymphoma and reactive follicular hyperplasia

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4. Follicular lymphomas
5. Morphology of follicular center B cell lymphoma
6. Burkitt lymphoma
7. Mycosis fungoides
8. Sézary syndrome

III. Multiple Choice Questions

1. **Majority of lymphoid neoplasms arise from**
   A. B cell
   B. T cell
   C. NK cell
   D. Monocyte
2. **The following marker is also known as leukocyte common antigen**
   A. CD10
   B. CD15
   C. CD30
   D. CD45
3. **All are B cell markers EXCEPT**
   A. CD 8
   B. CD10
   C. CD19
   D. CD21
4. **All the following are tumors of B cell EXCEPT**
   A. ALL
   B. CLL
   C. Mycosis fungoides
   D. Mantle cell lymphoma
5. **The most common cytogenetic abnormality of typical follicular lymphoma is**
   A. t(9;12)
   B. t(9;22)
   C. t(11;14)
   D. t(14;18)
6. **Molecular genetics in follicular lymphoma shows**
   A. Amplification of BAX protein
   B. Activation of telomerase
   C. Overexpression of BCL2 protein
   D. Deletion of RB gene
7. **The statement which is NOT true regarding Burkitt lymphoma is**
   A. Most of the tumors involve cervical and axillary lymph nodes
   B. Endemic (African) type of tumor is often associated with EBV infection
   C. Cytogenetically associated with t(8; 14) and overexpression of MYC protein
   D. On light microscopy it has a characteristic starry sky pattern

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8. **Mantle cell lymphoma is immunophenotypically negative for**
   A. Cyclin D1
   B. CD19
   C. CD20
   D. BCL6
9. **The neoplasm that does not arise from B cell is**
   A. Sézary syndrome
   B. Burkitt lymphoma
   C. Follicular lymphoma
   D. Small lymphocytic lymphoma
10. **Leukemic stage of T cell lymphoma is known as**
   A. Mycosis fungoides
   B. Sézary syndrome
   C. Adult T cell leukemia
   D. Hairy cell leukemia
11. **Hallmark cells are characteristic of**
   A. Hodgkin lymphoma
   B. Burkitt lymphoma
   C. Anaplastic large cell lymphoma
   D. Hairy cell leukemia
12. **Dutcher bodies are**
   A. Nuclear pseudo inclusions
   B. Cytoplasmic vacuoles
   C. Calcified concretions
   D. Giant mitochondria

ANSWERS

1. A **2.** D **3.** A **4.** C **5.** D **6.** C **7.** A **8.** D
9. A **10.** B **11.** C **12.** A

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CHAPTER 31 Hodgkin Lymphoma

CHAPTER OUTLINE

- Introduction
- Classical Hodgkin Lymphoma
- Nodular Lymphocyte Predominant Hodgkin Lymphoma
- Etiology and Pathogenesis of Hodgkin Lymphoma
- Laboratory Findings
- Clinical Features

INTRODUCTION

Definition

Hodgkin lymphomas (HL) are malignant lymphoid neoplasms which share the following characteristics:

- Heterogeneous cellularity comprising of **minority** (1 to 3%) **of specific neoplastic cells** (Hodgkin cells and Reed-Sternberg cells) in a **"characteristic background"** of reactive **non-neoplastic cells (majority)** of various types.
- The neoplastic cells are often ringed by T lymphocytes in a rosette-like manner.
- HL usually arises in lymphnodes (preferable site being cervical region).
- Majority manifest clinically in young adults.

Synonym: HL was recognized during 19th century by Thomas Hodgkin and was known as Hodgkin disease. Since the Reed-Sternberg cell originates from a lymphoid cell, the term Hodgkin lymphoma is preferred over Hodgkin disease.

Classification

The WHO classification (2008) recognizes two major types of HL (Table 31.1) namely classical HL and nodular lymphocyte predominant HL. These two types differ in clinical features, behavior, morphology and immunophenotype.

Table 31.1: WHO classification (2008) of Hodgkin lymphoma

- **Classical Hodgkin lymphoma**
  - **Nodular sclerosis classical Hodgkin lymphoma (NSCHL)**
  - **Mixed cellularity classical Hodgkin lymphoma (MCCHL)**
  - **Lymphocyte-rich classical Hodgkin lymphoma (LRCHL)**
  - **Lymphocyte depleted classical Hodgkin lymphoma (LDCHL)**
- **Nodular lymphocyte predominant Hodgkin lymphoma (NLPHL)**

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Cell of Origin and Immunophenotype

- **Classical Hodgkin lymphoma**
  - **Cell of origin:** In most cases of **classical HL,** the cell of origin of neoplastic cells (Hodgkin cells, Reed-Sternberg cells and its variants) is a **germinal center or post-germinal center B cell.**
  - **Immunophenotype: Classical forms of HL** (nodular sclerosis, mixed cellularity, lymphocyte-rich, and lymphocyte depletion) are characterized by Reed-Sternberg cells of similar immunophenotype, i.e. **CD15 and CD30 positive.**
- **Nodular lymphocyte predominant Hodgkin lymphoma**
  - **Cell of origin: Lymphocyte predominant** variant of Reed-Sternberg cell found in **nodular lymphocyte predominant HL,** arises from **germinal center B cell at the centroblastic stage** of differentiation.
  - **Immunophenotype:** The **lymphocyte predominant** variant of the Reed-Sternberg cells has an immunophenotype distinct from that of the classical HL subtypes. They are **CD15 and CD30 negative.**

Morphology of Neoplastic Cells

Reed-Sternberg (RS) Cells

- The neoplastic cells in Hodgkin lymphoma are **Reed-Sternberg cells** (Fig. 31.1) and its variant (Fig. 31.2). They are **pathognomonic** of Hodgkin lymphoma and histological diagnosis of HL should not be made in their absence. On the contrary, diagnostic Reed-Sternberg cells

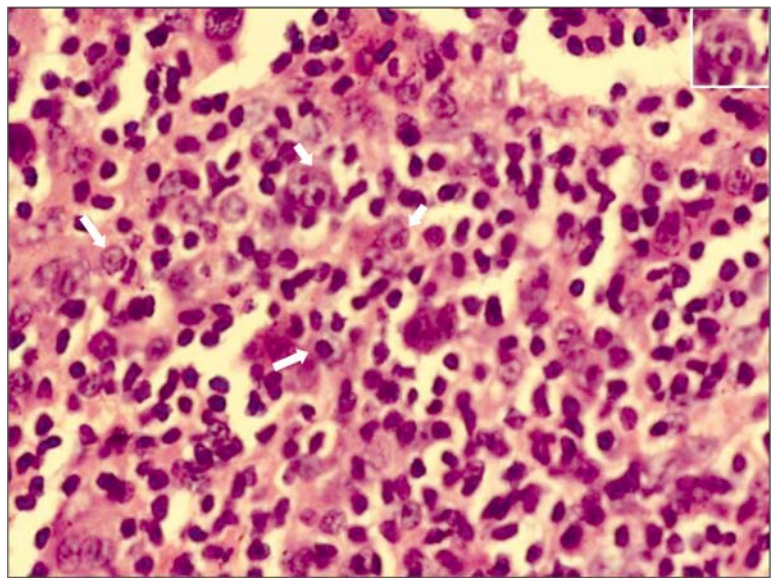


Fig. 31.1: Microscopic appearance of Hodgkin lymphoma showing RS cells (short arrow and inset) and Hodgkin cells (long arrow) within the background of mixed population of reactive cells

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are necessary but not sufficient for diagnosis of classical Hodgkin lymphoma. They should be found in a cellular background appropriate for the specific subtype of HL.

- **Appearance of diagnostic Reed-Sternberg cells:**
  - **Size:** RS cells are large cells ranging from 20 to 60 μm in diameter.
  - **Cytoplasm:** They have abundant eosinophilic cytoplasm.
  - **Nucleus:**
    Number: Typically they have **two large nuclei,** sometimes symmetric and the two nuclear lobes face each other (**"mirror image"**). The resulting appearance is often cited as an" **owl-eye" appearance**. However, the nucleus may be **single with multiple interconnected nuclear lobes or multiple nuclei**. When the nucleus is multilobated it has been termed as an "egg basket" appearance.
    Nuclear membrane: It is **thick** due to margination of chromatin.
    Nucleolus: Each nucleus or nuclear lobe has a single, **prominent inclusion-like eosinophilic nucleolus** about the size of a red cell or small lymphocyte (5–7 μm in diameter), often **surrounded by a halo.**

They are found in all classical subtypes of HL but not in nodular lymphocyte predominant type.

Reed-Sternberg Cell Variants (Fig. 31.2)

In addition to diagnostic RS cells, several variants have been recognized.

- **Mononuclear-variant:** It is also called as **Hodgkin cell** and is similar to the diagnostic RS cell except that it has a **single, large, round nucleus** with a large **eosinophilic inclusion-like nucleolus.** It may be found in any subtypes of the classical HL.

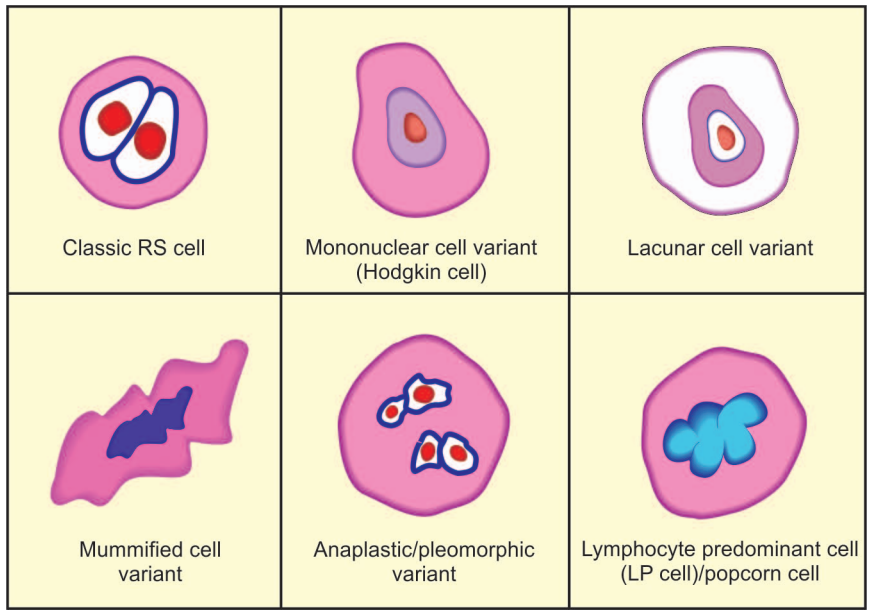


Fig. 31.2: Diagrammatic appearances of Reed-Sternberg cells and its variants

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- **Lacunar cell variant:** It is a variant of the Reed-Sternberg cell characteristically found in the **nodular sclerosis** classical Hodgkin lymphoma.
  - **Cytoplasm:** It has **abundant, lightly acidophilic or water-clear cytoplasm.**
  - **Nucleus:** Delicate large **folded or multilobed** nucleus.
  - **Nucleoli:** One or more **prominent eosinophilic nucleoli.**

  The lacunar appearance is a fixation artifact which causes retraction of cytoplasmic membrane during the formalin-fixed paraffin tissue processing. This creates an artificial space between the cytoplasm and peripheral cell margin. It is so named, as the nucleus appears to be sitting within a **space or 'lacuna'** (empty hole), caused by the shrinkage of the cytoplasm.
- **Mummified cell (necrobiotic) variant:** It is a shrunken, degenerated or apoptotic Reed-Sternberg cell.
  - **Cytoplasm:** This cell has **dark/deeply eosinophilic cytoplasm.**
  - **Nucleus: Nuclear detail is not preserved and appears pyknotic.**

  It may be seen singly or in clusters**.** They strongly indicate HL but are not diagnostic by themselves.
- **Anaplastic/pleomorphic variant:** This variant is commonly found in lymphocyte-depleted classical Hodgkin lymphoma.
  - **Size:** It is the largest type of RS cell and is highly **pleomorphic.**
  - **Nucleus:** This variant has **large, bizarre** and **polypoid hyperchromatic nucleus.**
  - **Nucleoli:** Prominent nucleoli**.**
- **Lymphocyte predominant cell (LP cell) /"popcorn" cell:** It is **specific to** the **nodular lymphocyte predominance type of HL.** It was formerly known as L and H cell (lymphocytic and/or histiocytic Reed-Sternberg cell variant).
  - **Cytoplasm:** It has relatively abundant and pale cytoplasm.
  - **Nucleus:** Delicate **multilobulated or folded nuclei** resembling bubbly outlines of popcorn kernels.
  - **Nucleoli:** One or more **inconspicuous nucleoli.**

Reed-Sternberg like cells: They can be found in conditions, such as:

- Infectious mononucleosis
- Metastatic carcinoma and melanoma
- Non-Hodgkin lymphoma
  - Peripheral T cell lymphoma
  - Anaplastic large cell lymphoma
  - T cell rich B cell lymphoma.

Diagnosis of HL: Reed-Sternberg cells are required for the diagnosis of HL, but they must be present in an appropriate background (Table 31.2) **of non-neoplastic inflammatory cells (lymphocytes, plasma cells, eosinophils).** Whenever diagnosis is difficult, immunohistochemical markers and molecular genetic studies will be of help in confirming the diagnosis.

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Table 31.2: Types of cells found in Hodgkin lymphoma

Non-neoplastic cells	*Neoplastic cells*
• Reactive lymphocytes • Macrophages/histiocytes ▪ Granulocytes ▪ Eosinophils ▪ Neutrophils • Plasma cells	• Reed-Sternberg cells (classical) • Variants ▪ Mononuclear ▪ Lacunar ▪ Mummified ▪ Anaplastic/pleomorphic ▪ Lymphocyte predominant (LP) cell/popcorn

General Morphological Features

Gross

- The affected lymph nodes are **enlarged** and have a smooth surface. Since, classical Hodgkin lymphoma (unlike the non-Hodgkin lymphoma) rarely breaches the lymph node capsule, on palpation they feel discrete.
- The cut surface is usually **homogeneously white**, although in some histological subtypes a nodular or fibrotic appearance may be present.

Microscopy

The affected lymph nodes show a **partial or complete effacement of their normal architecture** by a mixed infiltrate containing lymphocytes, histiocytes, plasma cells and eosinophils as well as the **characteristic RS cells or its variants**.

CLASSICAL HODGKIN LYMPHOMA

Classical Hodgkin lymphoma **(**CHL) is divided into **four subtypes**: nodular-sclerosis, mixed-cellularity, lymphocyte-rich, and lymphocyte-depleted variants. The immunophenotype and genetic features of the neoplastic cells are identical in these four histological subtypes, but their clinical features and association with EBV differs. They account for **95%** of all Hodgkin lymphomas.

Nodular Sclerosis Classical Hodgkin Lymphoma

Definition

Nodular sclerosis classical Hodgkin lymphoma **(NSCHL)** is a subtype of CHL characterized by **collagen bands** that surround **nodules** and have **lacunar cell variant of Reed-Sternberg cells.**

- Most common form of HL, constituting **40 to 70%** of cases.
- Most commonly occurs between **20–30 years of age** with **equal** frequency in **males and females.**

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Morphology

Sites involved

It usually involves the **mediastinal lymph nodes** and is only **rarely associated with EBV**. In the course of disease, it may involve the spleen, liver, bone marrow and other organs and tissues. A bizarre association of alcohol intolerance is often noted (5% of patients).

Microscopy

The normal lymph node architecture is replaced by cellular nodules surrounded by bands of collagen. It is characterized morphologically by the presence of:

- **Sclerosis and nodules (Fig. 31.3)*:*** Sclerosis is characterized by **broad, interconnecting collagen** bands that originate from the thickened lymph node capsule which divide the lymphoid tissue into **circumscribed nodules of varying sizes and shapes** (nodular sclerosis).
- **Lacunar cell:** Nodular sclerosis classical Hodgkin lymphoma is characterized by the presence of lacunar cell variant of Reed-Sternberg cell (Fig. 31.2). Diagnostic Reed-Sternberg cells are less frequent than in the mixed cellularity and lymphocyte depleted subtypes.

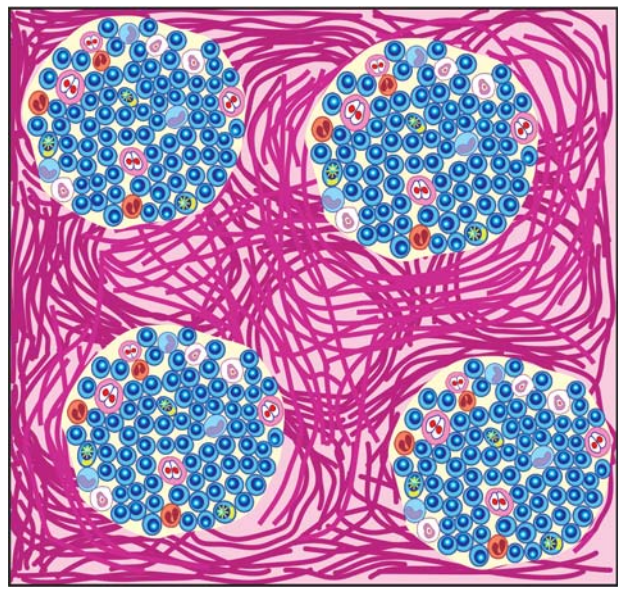


Fig. 31.3: Nodular sclerosis classical Hodgkin lymphoma with nodules separated by bands of collagen. Also seen are lacunar cells and RS cells in each nodules within the background of lymphocytes, eosinophils, plasma cells and macrophages

- **Background:** The neoplastic cells are found in a polymorphous background of **small T lymphocytes, eosinophils, plasma cells, and macrophages**. The cytological composition of the cellular nodules may vary from one in which the predominant cell is the small lymphocyte, with only scanty lacunar and Reed-Sternberg cells to a histological picture that is dominated by Hodgkin cells with depletion of lymphocytes.

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Immunophenotype

- The tumor cells have a characteristic immunophenotype. They are **positive for CD15 and CD30** and **negative for CD45 and T cell markers.**
- They are **usually EBV negative**.

Prognosis

Overall prognosis is **better than other types of CHL**, with a cure rate of 80 to 85%.

Mixed Cellularity Classical Hodgkin Lymphoma

Definition

Mixed cellularity classical Hodgkin lymphoma (MCCHL) is a subtype of classical Hodgkin lymphoma with scattered classical RS cells in a mixed inflammatory background without sclerosing fibrosis.

- Mixed cellularity is the **second common subtype** of classical Hodgkin lymphoma and constitutes about 20 to 25% of cases.
- Mixed cellularity HL is **more common in males** and **strongly associated with EBV**, as the Reed-Sternberg cells contain EBV genomes in at least 70% of cases.
- Compared to the lymphocyte predominance and nodular sclerosis subtypes, it is more likely to be associated with **older age**, **systemic symptoms** (such as night sweats and weight loss) and **advanced tumor stage.**

Morphology

Sites involved

It usually involves the **peripheral lymph nodes.**

Microscopy (Fig. 31.4)

- Lymph node architecture is usually obliterated, but the capsule is usually not thickened.
- **Diagnostic Reed-Sternberg cells** and mononuclear variants (Hodgkin cells) **are usually plentiful** (observed almost in every low-power field).
- **Back ground:** As the name suggests, the background shows **diffuse effacement** by a mixture of cell types, the composition of which varies. The cellular infiltrate consists of **small lymphocytes, eosinophils (sometimes numerous), neutrophils, plasma cells and benign macrophages (histiocytes).** Small lymphocytes in the background are predominantly T cells, and early disease involves the paracortical T cell zones in the lymph node.

Immunophenotype

It is identical to that observed in the nodular sclerosis type. The RS cells are **CD15+, CD30+** and EBV+ (about 70%).

Prognosis

Very good.

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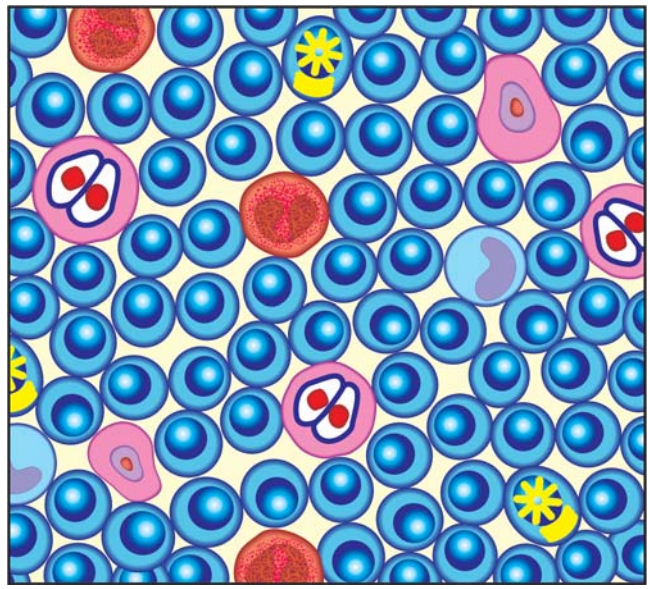


Fig. 31.4: Mixed cellularity classical Hodgkin lymphoma with classical RS cells, Hodgkin cells in the background of mixed cellular population consisting of lymphocytes, eosinophils, plasma cells and macrophages

Lymphocyte-Rich Classical Hodgkin Lymphoma

Definition

Lymphocyte-rich classical Hodgkin lymphoma (LRCHL) is a subtype of classical Hodgkin lymphoma with scattered Hodgkin and RS cells in a background consisting of small lymphocytes and with absence of neutrophils and eosinophils.

- These are an **uncommon** form and constitute about 5% of classical HL.
- It is more often seen in **elderly** patients, associated with **EBV in about 40%** of cases.

Morphology

Sites involved

Typically involves the **peripheral lymph nodes.**

Microscopy (Fig. 31.5)

- **Growth patterns**: It may show two growth patterns.
  - **Nodular** growth pattern is common
  - **Diffuse** growth pattern is rare
- Diagnostic ***Reed-Sternberg cells and mononuclear variants*** **are only few** in numbers**.**
- ***Background:*** As the name indicates the background consists of **abundant reactive small lymphocytes** with sparse or absent granulocytes.

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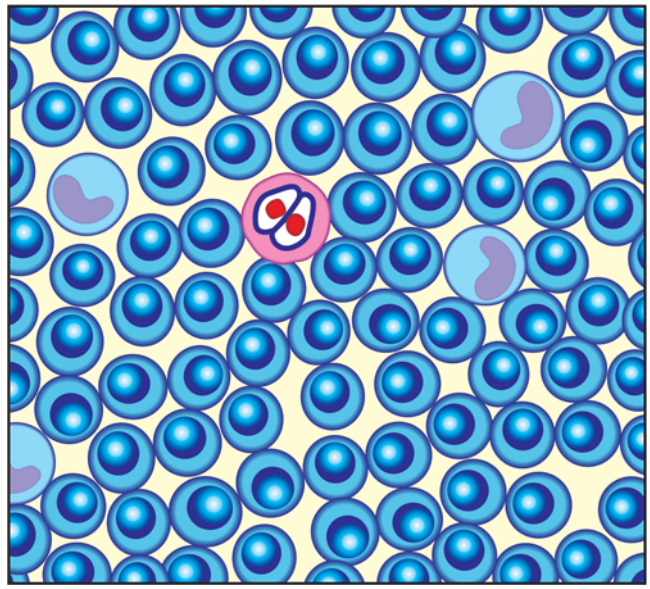


Fig. 31.5: Lymphocyte-rich classical Hodgkin lymphoma. One RS cell is seen in a background of many small lymphocytes and few histiocytes

Differential diagnosis

Entity is distinguished from the **nodular lymphocyte predominant** HL, in which the cells scattered among the lymphocytes are "popcorn"/LP cells. Immunophenotype typical of CHL is essential for its distinction from NLPHL.

Immunophenotype

It has a characteristic CD45–, CD20–, **CD15+ and CD30+** immunophenotype.

Prognosis

Good to excellent prognosis.

Lymphocyte-Depleted Classical Hodgkin Lymphoma

Definition

Lymphocyte-depleted classical Hodgkin lymphoma (LDCHL) is a diffuse subtype of classical Hodgkin lymphoma rich in Hodgkin and RS cells in a background depleted in non-neoplastic lymphocytes.

- Lymphocyte-depleted classical Hodgkin lymphoma is the **rarest** and constitutes less than 5% of cases.
- LDCHL is observed predominantly in **older patients**, **HIV-positive** individuals and is often **EBV-associated** (over 90%).

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Morphology

Sites involved

It involves predominantly **retroperitoneal lymph nodes, abdominal organs and bone marrow.**

Microscopy (Fig. 31.6)

- It is characterized by a **paucity of lymphocytes**.
- It shows a relative **abundance** of Reed-Sternberg cells or their **anaplastic/pleomorphic variants.**
- Two histological types are recognized.
  Reticular, which is characterized by numerous Hodgkin and Reed-Sternberg cells with depletion of lymphocytes.
  Diffuse sclerosis/fibrosis, where the lymph node architecture is replaced by a hypocellular infiltrate containing bizarre Reed-Sternberg cells and associated with non-collagenous fine fibrosis.

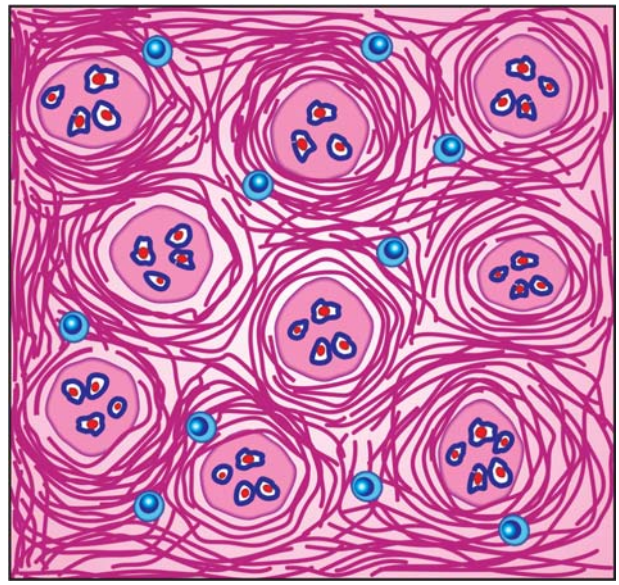


Fig. 31.6: Lymphocyte-depleted classical Hodgkin lymphoma with the pleomorphic variant of RS cells surrounded by fibrous tissue

Immunophenotype

The immunophenotype of the tumor cells is identical to that observed in the nodular sclerosis and mixed cellularity types. Thus, anaplastic RS cells are **CD15+, CD30+; majority are EBV+**. Phenotyping is critical for the diagnosis, since most tumors suspected of being lymphocyte depletion HL actually prove to be large-cell non-Hodgkin lymphomas.

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Prognosis

Advanced stage and systemic symptoms are frequent, and the overall outcome is somewhat **less favorable** than with other subtypes.

NODULAR LYMPHOCYTE PREDOMINANT HODGKIN LYMPHOMA

Definition

Nodular lymphocyte predominant Hodgkin lymphoma (NLPHL) is a monoclonal B cell neoplasm characterized by a nodular or a nodular and diffuse proliferation of scattered neoplastic cells known as popcorn or lymphocyte predominant cells (LP cells).

- It is an **uncommon variant**, accounting for approximately 5% of all Hodgkin lymphomas.
- NLPHL is **not associated** with **EBV** infection.
- Majority of the patients are males, usually in the **30–50 year** of age group.

Morphology

Sites Involved

Usually involves **cervical or axillary lymph nodes**. Mediastinal, splenic and bone marrow involvement is rare.

Microscopy (Fig. 31.7)

Growth pattern

- The lymph node architecture is completely or partially replaced by a nodular or a nodular and diffuse cellular infiltrate. Collagen band formation is not a feature.
- The cellular infiltrate consists of **abundant small lymphocytes** admixed with variable numbers of benign histiocytes and **scattered neoplastic cells** known as **lymphocyte predominant cells**.

Lymphocyte predominant cells (LP cells) /"popcorn" cells (Fig. 31.2):

- It is a distinctive RS cell variant and is specific to NLPHL. Typical **Hodgkin and Reed-Sternberg cell**s are extremely **difficult to find**.
- These cells are large with relatively abundant, pale cytoplasm.
- They have one large delicate **multilobulated** nucleus **or folded nuclei** resembling bubbly outlines of popcorn kernels.
- The nucleus has one or more **inconspicuous nucleoli.**

Background: It shows abundant small lymphocytes and variable number of macrophages (histiocytes). Other cells such as eosinophils, neutrophils and plasma cells are scanty or absent, and there is little evidence of necrosis or fibrosis.

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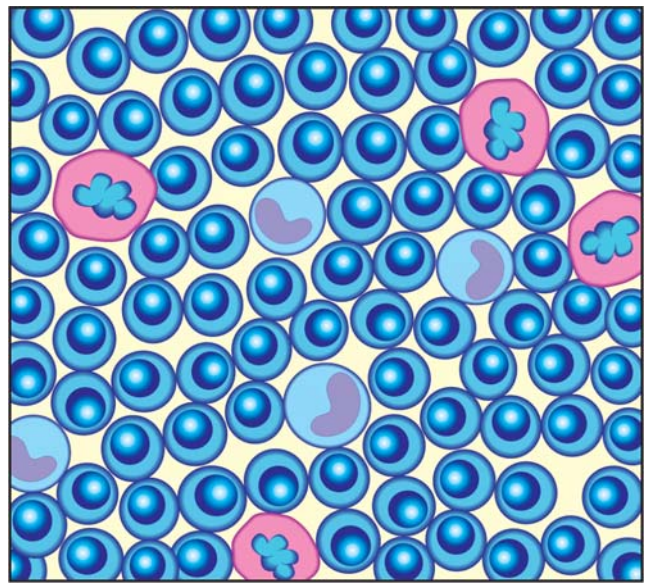


Fig. 31.7: Nodular lymphocyte predominant Hodgkin lymphoma with 'popcorn' cells in a background of reactive lymphocytes and few macrophages

Immunophenotype

Phenotypically and genotypically the popcorn cell (LP cell) has been shown to be derived from a neoplastic germinal center B cell. These RS cells are **CD20+**, **CD 45+** and **CD15–, C30– and EBV negative.** They express germinal center-specific transcription factor BCL6.

Cell of Origin

Germinal center B lymphocyte at the centroblastic stage of differentiation.

Transformation

About 3 to 5% of cases of nodular lymphocyte predominance HL transform to **diffuse large B cell lymphoma.**

Prognosis

It is more likely to **recur** than the classical subtypes, but the **prognosis is very good**.

ETIOLOGY AND PATHOGENESIS OF HODGKIN LYMPHOMA

- **EBV:** Young adults, who have had previous EBV infection (infectious mononucleosis), have an increased risk of developing HL and the EBV genome is frequently identified in the Reed-Sternberg cells.

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- **Genetic factors:** It may play a role because HLA subtypes, particularly HLA-B18, are higher in patients with HL.
- **Immune status:** HL seems to be more frequent in immunocompromised patients or those with autoimmune diseases, such as rheumatoid arthritis.

Pathogenesis (Fig. 31.8)

Relatively little is known about the factors that contribute to their neoplastic transformation.

- **EBV and HL**: It was observed that in HL associated with EBV, the infection by EBV occurred prior to cellular transformation. One of the EBV gene products LMP1 activates signaling pathways that promote B cell survival and proliferation. LMP1 prevents apoptosis and may act as an oncogene (Chapter 20).
- **Activation of nuclear factor:** NF-κB is a nuclear transcription factor that regulates expression of a large number of genes and one of them is the regulation of apoptosis. Hence, inappropriate **activation of NF-κB appears to be a common event** in classical HL. The activation of NF-κB may rescue germinal (post-germinal) center B cells from apoptosis and **produce Reed Sternberg cells.** The mechanisms of **activation of NF-κB** are:

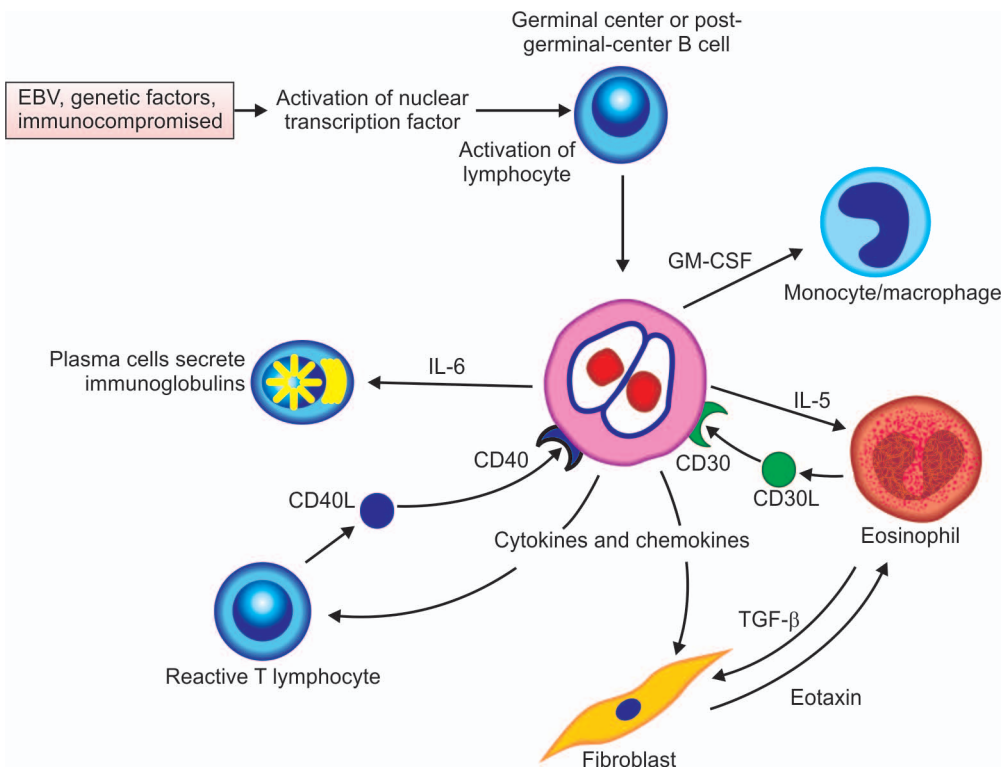


Fig. 31.8: Pathogenetic mechanism and interaction of various cell types in Hodgkin lymphoma

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- In **EBV-positive tumor,** the **LMP-1** transmits signals that **activate NF-κB.**
- In **EBV-negative** tumor; it may be due to **acquired mutations** in a negative regulator of NF-κB.

- **Accumulation of reactive cells:** The accumulation of reactive non-neoplastic cells in classical HL occurs in response to *cytokines* (such as IL-5, IL-6 and TGF-β) and *chemokines* that are secreted by Reed-Sternberg cells.
- **Actions of cytokines and chemokines:** They cause:
  - Clustering of CD4+ T cell lymphocytes around RS cells.
  - Recruitment of plasma cells (by IL-6) and associated immunoglobulin secretion by these cells.
  - Stimulation of fibroblasts.
  - Attraction of eosinophils by eotaxin which is a chemokine strongly expressed by fibroblasts. In turn, eosinophils are major source of fibrosis-promoting cytokine transforming growth factor (TGF-β).

  Once attracted by cytokines, the reactive infiltrate in turn supports the growth and survival of tumor cells (RS cells).
- **GM-CSF factor**: This factor from RS cell is responsible for accumulation of monocytes/macrophages.
- **Ligand for the CD30 and CD40 receptor**: Reactive eosinophils and T cells express ligand for the CD30 and CD40 receptor (present on the Reed Sternberg cells), respectively. They produce signals that lead to the activation of NF-κB.

LABORATORY FINDINGS

Peripheral Blood

- **Peripheral smear**
  - **RBCs:** Normocytic normochromic anemia is usually seen. Leukoerythroblastic picture may develop with bone marrow infiltration.
  - **WBCs:** Leukocytosis occurs in 1/3rd of the patients. Eosinophilia is frequent. In the advanced stage, lymphopenia with loss of cell-mediated immunity may develop.
  - **Platelets:** They are either normal or increased during early stage and may be reduced in later stages.
- **ESR:** Usually raised
- **Bone marrow:** Bone marrow may be involved in the later stages.

Biochemical Findings

- **C-reactive protein:** Usually increased
- **Serum lactate dehydrogenase (LDH):** It is **raised** in 1/3rd of cases.
- **Serum transaminases: May be raised** when liver is involved.

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Fine Needle Aspiration Cytology (FNAC)

FNAC of the involved lymphnode shows RS cells/its variants against a background of inflammatory cells (depending on the subtype).

Lymphnode Biopsy

Histopathological examination shows the characteristic morphological features, depending on the subtype.

CLINICAL FEATURES

HL usually presents with **painless enlargement of lymph nodes**. Most of the cases show cutaneous anergy due to depressed cell-mediated immunity. This suggests that HL arises in the background of an underlying immune abnormality.

Systemic/constitutional symptoms: These include fever, night sweats and weight loss which are mainly due cytokines and chemokines produced by tumor cells.

Spread: HL has a uniform pattern of remarkably predictable spread. It spreads mainly **by contiguity**, from one chain of lymph nodes to another. First it is nodal disease, then splenic disease, hepatic disease, and finally marrow involvement and extranodal disease. This is the reason for the cure with local radiotherapy in patients with limited disease.

Staging of HL (Table 31.3): It involves careful physical examination and investigative procedures, like radiologic imaging of the abdomen, pelvis and chest, and biopsy of the bone marrow. Staging is not only predictive of prognosis but also guides the choice of therapy. With current treatment protocols, tumor stage rather than histological type is the most important prognostic variable. The cure rate of patients with stages I and IIA is close to 90%. Even with advanced disease (stages IVA and IVB), 60 to 70% 5-year disease-free survival is obtained.

Table 31.3: Clinical staging of Hodgkin lymphomas (Cotswold revision of Ann Arbor staging classification)

Stage	*Definition*
I	Involvement of a single lymph node region or lymphoid structure (e.g. spleen, Waldeyer ring, thymus)
II	Involvement of two or more lymph node regions on the same side of the diaphragm (the mediastinum is a single site; hilar lymphnodes are lateralized); the number of anatomic sites should be indicated by suffix (e.g. II_3)
III	Involvement of lymph node regions or structures on both sides of the diaphragm III_1 With or without splenic, hilar, celiac or portal nodes III_2 With para-aortic, iliac or mesenteric nodes
IV	Involvement of extranodal site(s) beyond those designated E

E-involvement of a single extranodal site, or contiguous or proximal to known nodal site of disease.

Sequelae: Long-term survivors of HL following chemotherapy and radiotherapy have an increased risk of developing myelodysplastic syndromes, acute myelogenous leukemia (AML),

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lung cancer, NHL, breast cancer, gastric cancer, sarcoma and malignant melanoma. Carcinoma and other solid tumors follow radiotherapy, whereas chemotherapeutic drugs increase the risk of acute myelogenous leukemia and myelodysplasia.

HL differs from NHL in several respects and their main differences are shown in Table 31.4.

Table 31.4: Differences between Hodgkin and Non-Hodgkin lymphomas

	Characteristics	*Hodgkin Lymphoma*	*Non-Hodgkin Lymphoma*
1	Site of involvement	Arises in a single node or chain of nodes (cervical, mediastinal, para-aortic)	Mainly involves multiple peripheral nodes
2	Pattern of spread	Orderly spread by contiguity in a predictable fashion	Noncontiguous spread in an unpredictable fashion
3	Mesenteric nodes and Waldeyer ring	Rarely involved	Commonly involved
4	Extranodal involvement	Uncommon	Common
5	Characteristic of neoplastic cells	Neoplastic cells—Hodgkin or Reed-Sternberg cells form minor tumor cell mass (1 to 5%)	Neoplastic cells form the major tumor cell mass

SUMMARY

- Hodgkin lymphoma is a malignant lymphoid neoplasm characterized by a heterogeneous cellularity comprising of minority of neoplastic cells (Hodgkin cells and RS cells) in a background of majority of reactive cells.
- WHO classifies HL into classical and nodular lymphocyte predominant. Classical HL is subclassified as nodular sclerosis, mixed cellularity, lymphocyte-rich and lymphocyte-depleted.
- Neoplastic cells in Hodgkin lymphoma are Reed-Sternberg cells and its variants. They are pathognomonic of Hodgkin lymphoma and are derived from germinal center or immediate post-germinal center B cells.
- Typically RS cells have abundant eosinophilic cytoplasm with two nuclear lobes facing each other ("mirror image"), prominent eosinophilic nucleoli resulting in an "owl-eye" appearance.
- The variants of RS cells include mononuclear, lacunar, anaplastic, mummified cell and popcorn types.
- Classical HL: RS cells in all the classical HL are CD15+, CD30+; whereas in nodular lymphocyte predominant they are negative with these markers but CD20+.
  - Nodular sclerosis classical HL is the most common subtype with frequent mediastinal involvement. It is characterized by fibrous bands dividing cellular areas into nodules. Lacunar cells are frequent with occasional diagnostic RS cells.
  - Mixed cellularity classical HL shows mononuclear and diagnostic RS cells within the background of mixed infiltrate of reactive T lymphocytes, eosinophils, macrophages and plasma cells.

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  - Lymphocyte rich classical HL is uncommon and diagnostic RS cells are seen in a background rich in reactive T lymphocytes.
  - In lymphocyte depletion classical HL, diagnostic RS cells and anaplastic/pleomorphic variants are seen in a background with paucity of lymphocytes.
- Nodular lymphocyte predominant is uncommon and consists of popcorn cell in the background of reactive B cells and benign histiocytes. RS cells are CD20+, CD15–, C30– and EBV-.

SELF-ASSESSMENT EXERCISES

I. Essays

1. Classify Hodgkin lymphoma. Describe the gross and microscopic features of all subtypes. Compare their prognosis.
2. Mention the WHO classification of Hodgkin lymphoma. Discuss the subtypes of Hodgkin lymphoma.

II. Short Notes

1. Gross and microscopy of Hodgkin lymphoma
2. Reed-Sternberg cell and its variants
3. Nodular sclerosis type of Hodgkin lymphoma/gross and microscopic features of nodular sclerosis type of Hodgkin lymphoma
4. Mixed cellularity Hodgkin lymphoma/gross and microscopic features of mixed cellularity type of Hodgkin lymphoma
5. Gross and microscopic features of lymphocyte predominant type of Hodgkin lymphoma
6. Differences between Hodgkin and Non-Hodgkin lymphoma

III. Multiple Choice Questions

1. **Percentage of neoplastic cells in Hodgkin lymphoma is in the range of**
   A. 1 tp 3
   B. 10 to 15
   C. 40 to 50
   D. 80 to 90
2. **The cell which is NOT found in Hodgkin lymphoma is**
   A. Lacunar cell
   B. Mummified cell
   C. LP cell
   D. Langerhans cell
3. **The following are the subtypes of classical Hodgkin lymphoma EXCEPT**
   A. Nodular lymphocyte predominant
   B. Nodular sclerosis
   C. Mixed cellularity
   D. Lymphocyte depleted

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4. **The subtype of the Hodgkin lymphoma is immunophenotypically negative for CDl5 and CD30 is**
   A. Nodular sclerosis
   B. Nodular lymphocyte predominant
   C. Lymphocyte depleted
   D. Mixed cellularity
5. **The subtype of Hodgkin lymphoma that shows lacunar cells and collagen bands is**
   A. Nodular lymphocyte predominant
   B. Lymphocyte depleted
   C. Mixed cellularity
   D. Nodular sclerosis
6. **Reed-Sternberg cells are found in**
   A. Hodgkin lymphoma
   B. Burkitt lymphoma
   C. Follicular lymphoma
   D. Diffuse lymphoma
7. **The subtype of Hodgkin lymphoma NOT associated with EBV is**
   A. Nodular lymphocyte predominant
   B. Lymphocyte depleted
   C. Mixed cellularity
   D. Lymphocyte rich
8. **The subtype of Hodgkin lymphoma associated with popcorn cell is**
   A. Nodular lymphocyte predominant
   B. Lymphocyte depleted
   C. Mixed cellularity
   D. Nodular sclerosis
9. **All are true about Hodgkin lymphoma EXCEPT**
   A. Bimodal age pattern
   B. Less aggressive than NHL
   C. Contiguous spread
   D. Osteolytic metastasis

ANSWERS

1. A	**2.** D	**3.** A	**4.** B	**5.** D
6. A	**7.** A	**8.** A	**9.** D	

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CHAPTER 32 Langerhans Cell Histiocytosis

CHAPTER OUTLINE

- Introduction
- Definition
- Morphology
- Laboratory Findings

INTRODUCTION

Histiocytic and dendritic cell neoplasms are group of proliferative disorders derived from the mononuclear phagocytes (macrophages and dendritic cells).

Langerhans cell: It is a myeloid stem cell derived dendritic cell. They are found in the epidermis, lymph nodes, spleen, thymus and mucosal tissues. Langerhans cells are antigen presenting cells which ingest, process and present antigens to T lymphocytes, and thus play an important role in immunity. In lymph nodes, Langerhans cells are known as interdigitating reticulum cells (IDCs).

Langerhans cell histiocytosis is one of the tumors under the category of histiocytic and dendritic cell neoplasms (WHO classification 2008).

DEFINITION

Langerhans cell histiocytosis (LCH)/ Histiocytosis X is a clonal proliferative disorder arising from Langerhans cells.

Sites involved: Depending on the site involved and distribution of lesion, LCH can be divided into three groups (Table 32.1).

The combination of calvarial bone defects, diabetes insipidus and exophthalmos is called as the **Hand-Schüller-Christian triad.**

Etiology and pathogenesis: It is **unknown.**

MORPHOLOGY

The important feature is the identification of Langerhans cells.

- ***Light microscopy:*** The characteristic features of **Langerhans cells** are:

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Table 32.1: Types of Langerhans cell histiocytosis

Terminology/site	Involved tissue/organ	Clinical features
Eosinophilic granuloma Localized to a single site/ solitary (unifocal)	Bone and adjacent soft issue (skull, femur, vertebra, pelvic bones and ribs). Less commonly lymph nodes	Usually seen in older children or adults. Presents with lytic bone lesion
Hand-Schüller-Christian disease Multiple sites within a single system (multifocal unisystem)	Usually bone and soft issue	Usually seen in young children Multiple destructive bone lesions with adjacent soft tissue masses
Letterer-Siwe disease Disseminated and multisystemic disease (multifocal multisystem disease)	Skin, bone, liver, spleen and bone marrow	Usually seen in infants. Present with fever, cytopenias, skin and bone lesions and hepatosplenomegaly

- **Size**: Large 10-15 µm in diameter
- **Cytoplasm:** Moderately abundant and slightly eosinophilic
- **Nucleus:**
  - **Appearance:** Folded, indented, grooved or lobulated
  - **Chromatin:** Fine chromatin
  - **Nucleoli:** Inconspicuous

Background: The Langerhans cells are usually seen in a mixed background of eosinophils, mononuclear and multinuclear histiocytes, neutrophils and small lymphocytes.

- ***Electron microscopy*:** Langerhans cell contains **pathognomonic Birbeck granules**, which are distinctive rod-shaped or tubular cytoplasmic inclusions with a dense core and a double outer sheath. Frequently, one end of the granule is dilated (bulbous), producing a **tennis racket–like shape, with a zipper-like appearance**.
- ***Immunological markers:*** Langerhans cells express CD1a, langerin and S-100 protein. In addition, the tumor cells also typically express HLA-DR.

LABORATORY FINDINGS

- ***Peripheral blood:*** Extensive infiltration of the marrow causes pancytopenia (anemia, neutropenia and thrombocytopenia) and a predisposition to recurrent infections, such as otitis media and mastoiditis.
- ***Bone marrow:*** It shows extensive infiltration by histiocytes.

Prognosis: Depends on the age at presentation, extent of disease and rate of progression.

SUMMARY

- Langerhans cell histiocytosis (LCH) is a spectrum of clonal proliferative disorders of Langerhans cell.
- The Langerhans cell on electron microscopy contains pathognomonic Birbeck granules.

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- LCH spectrum ranges from unifocal to multifocal and unisystem to multisystem disease.
- Microscopically, it shows proliferations of Langerhans cells along with eosinophils, lymphocytes, plasma cells and neutrophils.

SELF-ASSESSMENT EXERCISES

I. Short Note

Langerhans cell histiocytosis

II. Multiple Choice Questions

1. **All the following disorders belong to the group of Langerhans cell histiocytosis EXCEPT**
   A. Eosinophilic granuloma
   B. Chronic granulomatous disease of childhood
   C. Hand-Schüller-Christian disease
   D. Letterer-Siwe disease
2. **The characteristic feature of Langerhans cell is the presence of**
   A. Birbeck granules
   B. Auer rods
   C. Owl-eyed nuclei
   D. CD15 positive histiocytes

ANSWERS

1. B **2.** A

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SECTION 3

Disorders of Hemostasis

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CHAPTER 33

Normal Hemostasis and its Components

CHAPTER OUTLINE

- Introduction
- Platelets
- Blood Vessel Wall
- Coagulation System
- Coagulation Regulatory Mechanism
- Fibrinolytic System
- Normal Hemostasis

INTRODUCTION

Definition

Hemostasis is a regulated process by which blood is maintained in liquid state inside the vessels, yet arrests bleeding at the site of vascular injury by forming a hemostatic clot.
Thrombosis is a pathological counterpart of hemostasis in which blood clot (thrombus) is formed inside the circulation.

Components of normal hemostasis and thrombosis: Five components namely: **platelets, blood vessel wall (endothelium), coagulation system, coagulation regulatory system and fibrinolytic system** participate both in hemostasis and thrombosis.

PLATELETS

- Platelets are produced from bone marrow precursors known as megakaryocytes.
- Normal platelet count ranges from 1, 50,000 – 4, 00,000/cu mm (150–400 × 10^9/L) and it has a normal life span of 8-10 days.

Morphology of Platelets

- **Light microscopy:** On Romanowsky-stained peripheral blood smear, platelets appear as small (diameter of 2-3 µm, approximately 1/5 the diameter of red blood cell), round, anuclear cells with prominent reddish purple granules.
- **Electron microscopy:** Platelets (Fig. 33.1) reveal several glycoprotein receptors, two types of cytoplasmic granules and a contractile cytoskeleton.
  - **Glycoprotein receptors:** The two main platelet membrane receptors are:
    - ***Glycoprotein (Gp)* IIb-IIIa:** It is the main receptor on cell surface.
    - ***Glycoprotein* Ib-IX:** It is a receptor for binding vWF with platelets.

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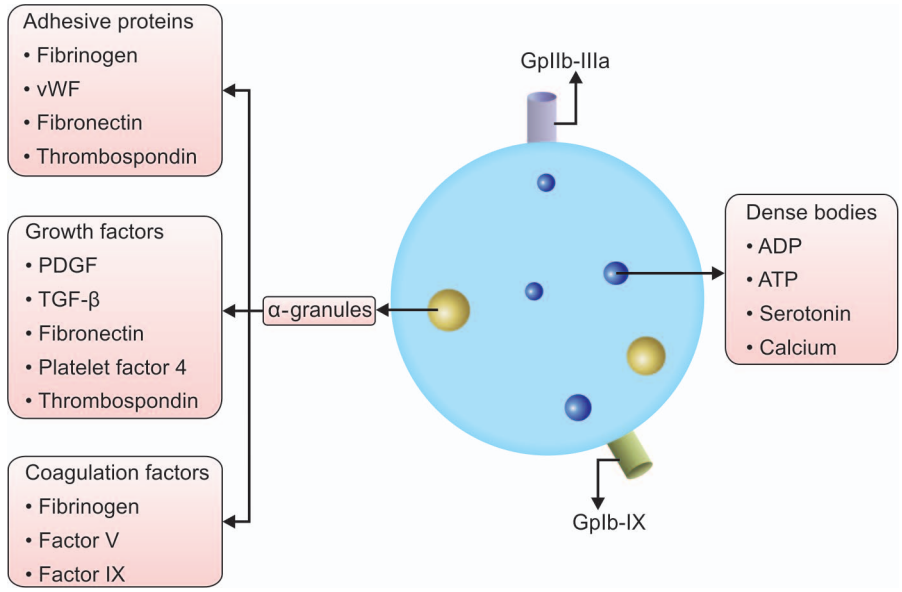


Fig. 33.1: Cellular constituents of platelets under electron microscope

- **Cytoplasmic granules**
  - **Alpha (α) granules** contain fibrinogen, factors V and VIII, von Willebrand factor (vWF), fibronectin, chemokines, platelet factor 4 (heparin-binding chemokine), platelet-derived growth factor (PDGF) and transforming growth factor-β (TGF-β). They also have adhesion molecule P-selectin on their membranes.
  - **Dense granules** contain potent aggregating molecules adenosine diphosphate (ADP), ATP, serotonin, ionized calcium, histamine and epinephrine.
- **Contractile cytoskeleton:** It consists of dense microtubules and circumferential microfilaments, which maintain the disk shape of platelets.

Role of Platelets in Hemostasis

- Form the primary hemostatic plug.
- Release platelet activating and procoagulant molecules.
- Provide a procoagulant surface for the activation of coagulation system.

BLOOD VESSEL WALL

Endothelial Cells

- In the blood vessel wall, particularly endothelial cells play an important role in the regulation of homeostasis. The normal intact smooth endothelial cells lining the blood vessels inhibit

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platelet activation, prevent coagulation and enhance fibrinolysis. The **endothelial cell, thus presents as a nonthrombogenic** surface and maintains blood in a fluid state. Endothelial cells use three antithrombotic systems (Fig. 33.7) to limit coagulation. These are (1) antithrombin III, (2) protein C and protein S, (3) tissue factor pathway inhibitor (TFPI).

- The injured endothelium or under inflammatory conditions, it downregulates its anticoagulant functions and becomes procoagulant. **Injury to endothelial cells or endothelial activation exposes highly thrombogenic subendothelial extracellular matrix** (ECM), which leads to platelet adherence and activation. There is simultaneous activation of coagulation system.

Vasoconstriction

- Following injury, blood vessels also undergo vasoconstriction which assists in arresting the blood loss. Vasoconstrictors may also be produced by endothelial cells (angiotensin II) and activated platelets (thromboxane A_2).

COAGULATION SYSTEM

Coagulation factors or clotting factors are transported in the plasma as procoagulants. During coagulation, the procoagulants become activated to produce **thrombin**. Thrombin converts the soluble **fibrinogen** into insoluble meshwork of **fibrin gel**.

Note: When procoagulant clotting factor becomes activated, it is designated by a lower-case letter "a" after the numerical. (e.g. activated factor V is Va).

Traditional Coagulation Pathways (Fig. 33.2 and Table 33.1)

Coagulation can be activated by two pathways namely *extrinsic* and *intrinsic*. Both the pathways converge on the activation of factor X.

Extrinsic Pathway

- It requires an exogenous trigger; hence the name extrinsic, but now it is observed that released **tissue factor** from vascular injury can initiate this pathway.
- Tissue factor is present on the vascular wall and is not normally in contact with blood. Whenever there is **vascular injury,** the **tissue factor** (also known as thromboplastin or factor III) is liberated at the site and **triggers coagulation** by this pathway,
- The coagulation factor utilized in extrinsic pathway is factor VII.
- The **prothrombin time (PT)** is the laboratory test which assesses the function of the coagulation factors involved in the extrinsic pathway (factor VII) and common pathway (factors X,II, V and fibrinogen). PT is performed by adding tissue factor.

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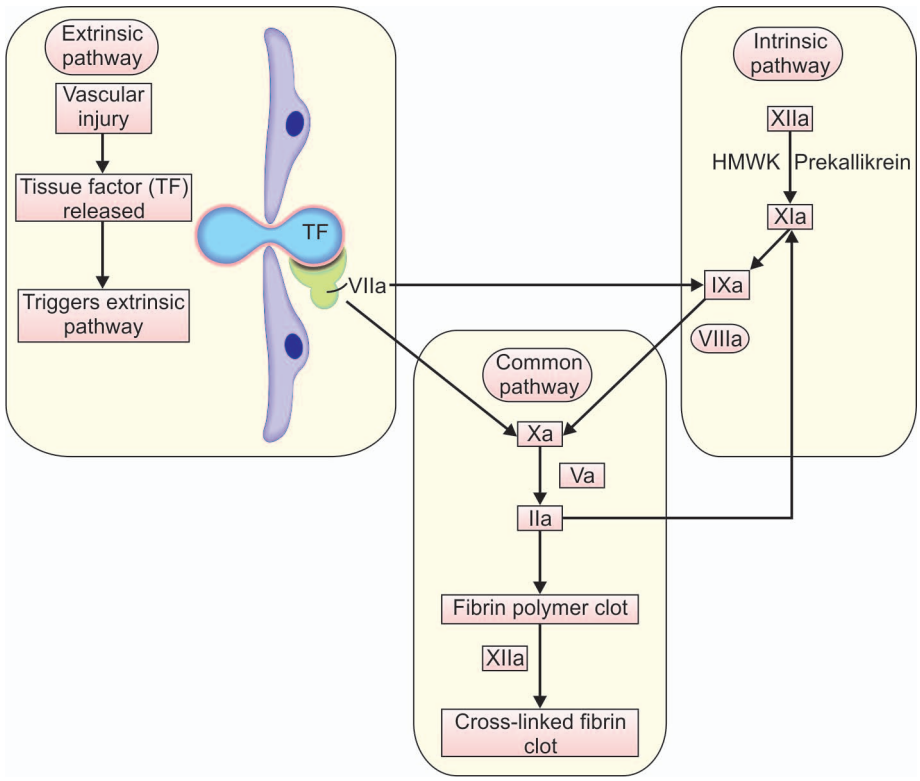


Fig. 33.2: Different pathways of coagulation (extrinsic, intrinsic and common) pathways

Table 33.1: Coagulation factors involved in different pathways

Extrinsic pathway	*Intrinsic pathway*	*Common pathway*
VII	XII Prekallikrein HMWK XI IX VIII	X V Prothrombin Fibrinogen

Intrinsic Pathway

- It is activated by exposure of factor XII (Hageman factor) to any thrombogenic surfaces (including glass beads).
- The coagulation factors utilized in intrinsic pathway in order of reaction are: factors XII, prekallikrein HMWK, XI, IX and VIII (Table 33.1).
- The **partial thromboplastin time (PTT)/activated partial thromboplastin time (APTT)** assesses the function of the coagulation factors utilized in the intrinsic pathway (factors XII, pre-K, HMWK, XI, IX and VIII) and common pathway (factors X, V, II and fibrinogen). PTT is initiated by adding negatively charged particles like glass beads.

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Common Pathway

Above two pathways have in common, factors X, V, prothrombin and fibrinogen; and this part of coagulation pathway is known as the common pathway.

This division is arbitrary because there are many interconnections between these two pathways.

Cell-based Coagulation

Although the classical model of coagulation supports that many proteins take part *in vitro* coagulation cascade, but it does not adequately explain the mechanisms leading to hemostasis *in vivo* where only few proteins are involved. In particular, it does not explain why certain patients demonstrate a hemorrhagic tendency; nor does it allow accurate prediction of which patients will actually bleed. Currently, a new cell-based coagulation cascade model has been developed which (occurs *in vivo*) will replace the classical model of the coagulation cascade.

Steps

Cell-based coagulation (Fig. 33.3) occurs on different cell surfaces, in three overlapping steps:

- ***Initiation phase:***
  - Cell-based coagulation system becomes initiated on the surface of a tissue factor (TF)-bearing cell.
  - Tissue factor (TF) forms complex with activated factor VII (VIIa).
  - Factor X is activated by the TF: VIIa complex and generates thrombin (IIa). The quantity of thrombin generated is not enough to convert fibrinogen to fibrin.
- ***Amplification phase:*** In the amplification phase, platelets and co-factors are activated in order to prepare for large-scale thrombin generation. The little amount of thrombin (IIa) produced during initiation phase is enough only to activate the following:
  - Platelets: Activated platelets release factor V which is activated by thrombin to factor Va on the platelet surface.
  - Factor VIII which is freed from vWF complex and binds to platelet surface.
  - Factor XI
- ***Propagation phase:*** Propagation occurs on the surface of platelets and results in the propagation of large amounts of thrombin:
  - Factor IX is also activated by TF: VIIa complex and binds to factor VIIIa on platelet surface and activates factor X.
  - Factor IXa is not readily degraded by plasma inhibitors. So they continue activating factor X.
  - Activated factor Xa binds to factor Va on the platelet surface and activates prothrombin, producing enough quantity of thrombin to convert fibrinogen into fibrin.

COAGULATION REGULATORY MECHANISM

The activated coagulation system must be limited to the site of vascular injury so as to prevent coagulation in the entire vascular system. This is achieved by three endogenous anticoagulants.

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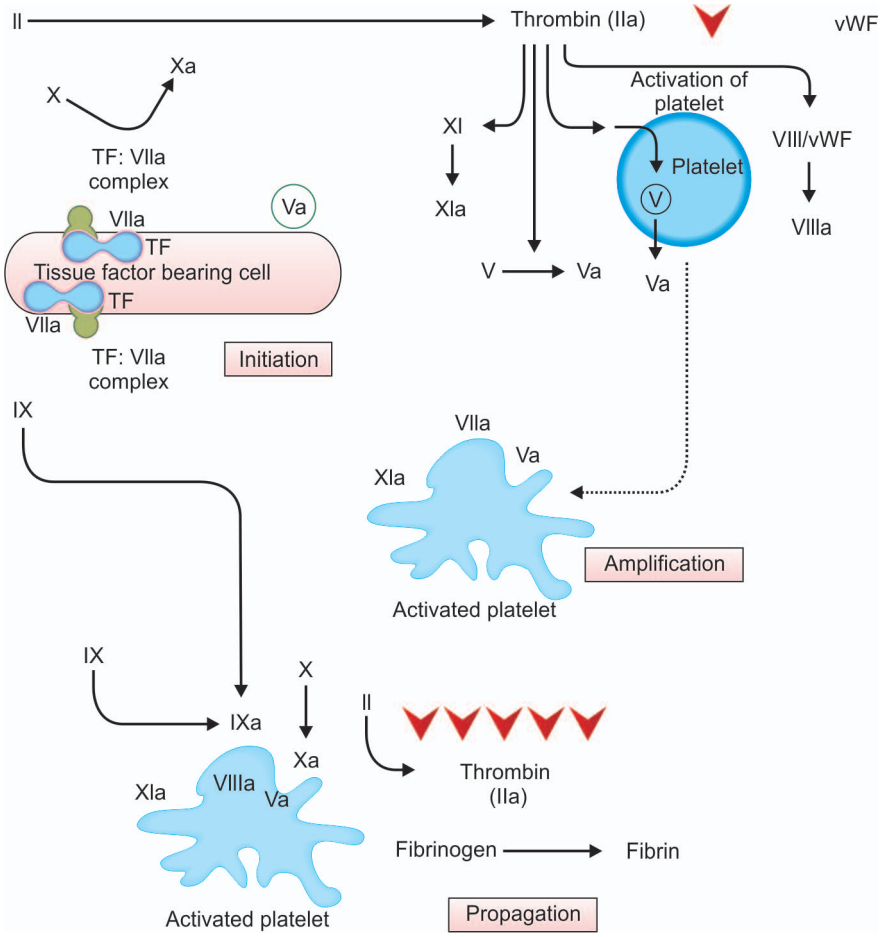


Fig. 33.3: Various steps involved in cell-based coagulation

- **Antithrombin:** It inhibits the activity of thrombin and factors IXa, Xa, XIa and XIIa. One of the antithrombin is antithrombin III which gets activated by binding to heparin-like molecules on endothelial cells. The heparin used to minimize thrombosis acts by activating antithrombin III (Fig. 33.7)
- **Proteins C and S:** These are vitamin K dependent proteins that act as a complex. Thrombin generated by activation of coagulation cascade, binds with thrombomodulin present on the endothelial cell membrane and activates the coagulation regulatory system called protein C system. Activated protein C (APC) binds with protein S to form APC-protein S complex. This complex inactivates factors Va and VIIIa (Figs 33.4 and 33.7)
- **Tissue factor pathway inhibitor (TFPI):** TFPI is a protein which inactivates tissue factor–factor VIIa complexes and inactivates factor VIIa (Figs 33.5 and 33.7).

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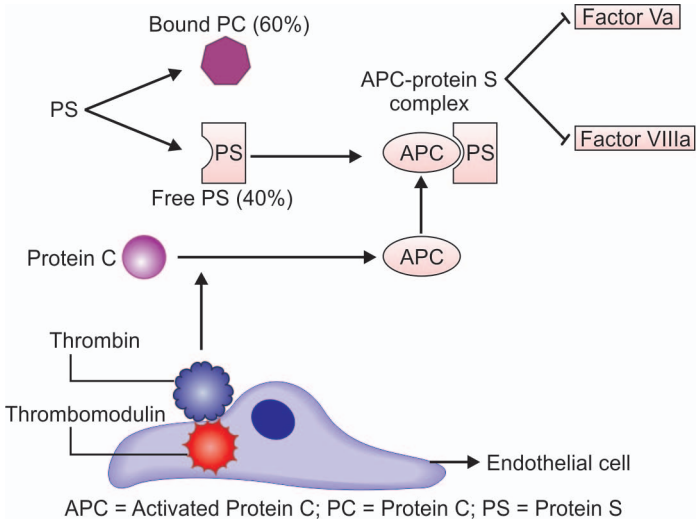


Fig. 33.4: Protein C and S pathway. Thrombomodulin from endothelial cell membrane binds to thrombin and activates protein C. Activated protein C (APC) combines with free protein S to form complex which inactivates active factor V and VIII

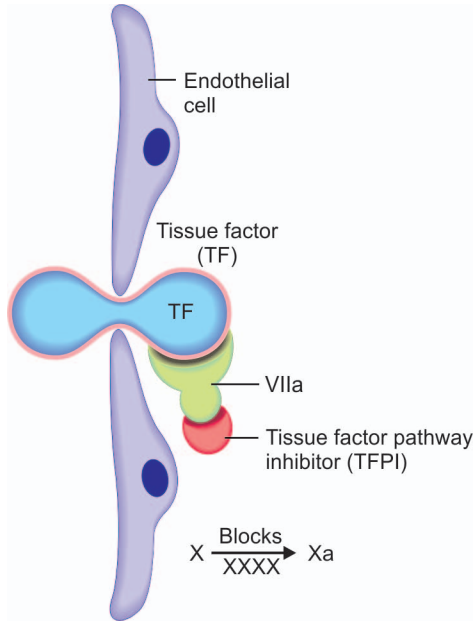


Fig. 33.5: TFPI inactivates tissue factor–factor VIIa complexes

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FIBRINOLYTIC SYSTEM

Activation of the coagulation also initiates fibrinolytic system (Fig. 33.6 and 33.7) so that the size of the clot is limited. Fibrinolytic system does this by removal of fibrin from the clot. Otherwise the clot may progress and involve the entire circulation with its consequences.

- In the fibrinolytic system, **plasmin is formed** from the inactive circulating precursor **plasminogen.**
  Plasminogen may be activated either by:
  - **A factor XII–dependent pathway** or
  - **Plasminogen activators (PAs):** The most important of the PAs is tissue plasminogen activator (t-PA) secreted by endothelial cells and others include **urokinase.**
- **Free plasmin** is rapidly **inactivated** by binding to a plasma protein named α_2-antiplasmin, thereby limiting the action of plasmin.
- **Plasmin cleaves fibrinogen and fibrin** and produces number of fibrin degradation (FDP) products, also known as fibrin split products (fragments like X, Y, D, E and D-D). These FDPs clear minor clots in the vessels and restore the blood flow. Elevated levels of FDPs, most notably **D-D fragment, known as D-dimers** is used as a marker for thrombosis and is used for the diagnosis of disseminated intravascular coagulation (DIC).
- The **activity of t-PA and urokinase** is **controlled by** releasing plasminogen activator inhibitor-1 **(PAI)** secreted by endothelial cells.

NORMAL HEMOSTASIS

Hemostasis is the body's response to vascular damage and includes several sequences of events at a site of vascular injury (Fig. 33.8). Normally, blood vessels are lined by a smooth, nonthrombogenic endothelium. **Injury to the endothelium** causes transient vasoconstriction and **exposes highly thrombogenic subendothelial extracellular matrix** (ECM), **initiating the platelet events.** These events forms hemostatic plug and prevents any further hemorrhage at the site of injury.

Primary Hemostatic Plug

Different events in platelet activation are:

- **Platelet adhesion and shape change:**
  - Initial step is **adhesion of platelets** to subendothelial structures at the site of injury. The link is mainly through receptor sites (GpIb-IX) on the platelet with subendothelial **von Willebrand factor (vWF).** vWF is synthesized by both endothelial cells and megakaryocytes.
  - **Platelets change their shape** from round to spherical to stellate, thereby markedly increasing the surface area.
- **Platelet secretion (release reaction):**
  - Soon after adhesion, platelets **release granule contents** which contain pro-aggregatory substances like ADP, serotonin (5-hydroxytryptamine), fibrinogen and vWF. Calcium is also released and is required for the coagulation.

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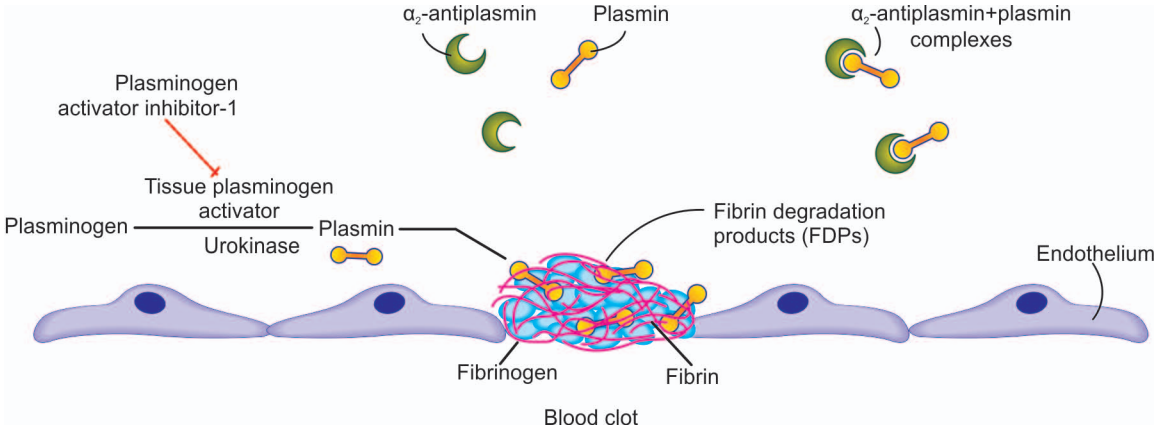


Fig. 33.6: Fibrinolytic system

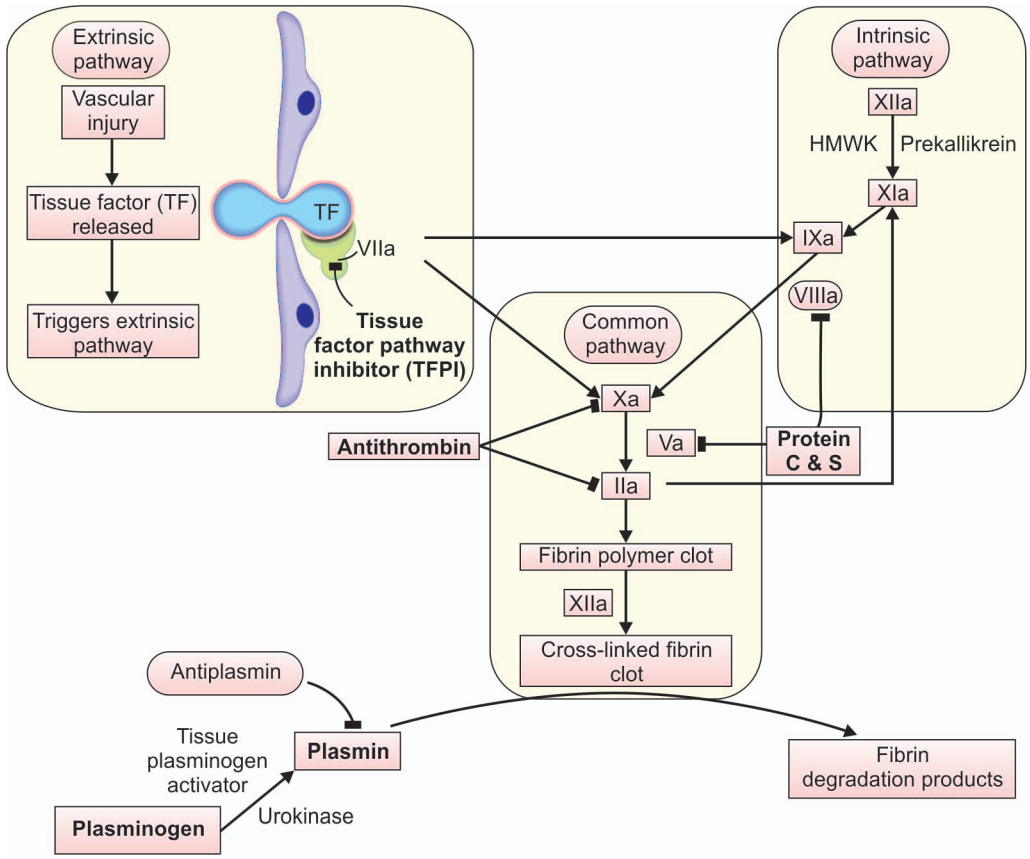


Fig. 33.7: Interactions among coagulation, coagulation regulatory and fibrinolytic system

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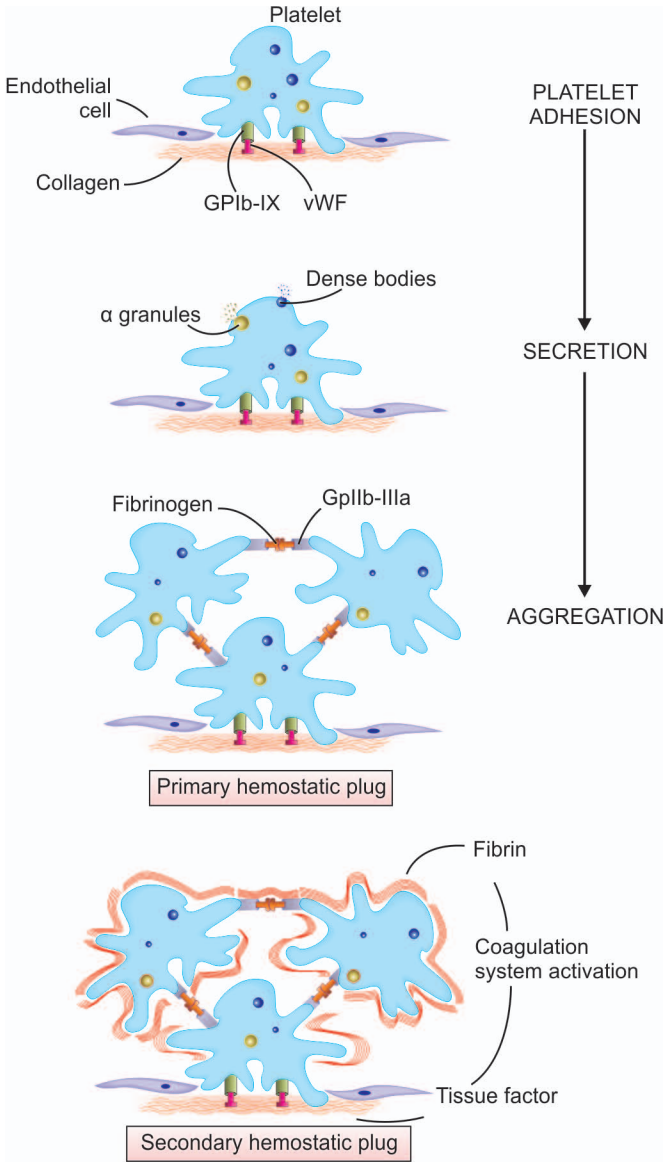


Fig. 33.8: Different steps in hemostasis

- **Platelet aggregation:**
  - The secreted products recruit additional platelets and cause **aggregation** to each other through the receptor sites (Gp IIb-IIIa) using fibrinogen as an intercellular bridge.
  - These clumps of platelets so formed quickly stop bleeding from the site of injury and are known as **primary hemostatic plug.** The process of primary hemostatic plug formation is termed as **primary hemostasis.**

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Secondary Hemostatic Plug

- **Exposure of tissue factor** at the site of injury **activates the extrinsic coagulation system.** The process generates thrombin, which cleaves circulating fibrinogen into insoluble **fibrin,** creating a fibrin meshwork at the injured site.
- The platelets and other circulating cells get trapped in the fibrin meshwork. The platelets contract, form an irreversibly fused mass and constitute definitive **secondary hemostatic plug.** The process of conversion of the initial temporary primary hemostatic (platelet) plug into a permanent secondary hemostatic plug is known as **secondary hemostasis.**
- The solid permanent plug so formed prevents any further hemorrhage. The hemostatic plugs/clots thus formed are confined to the site of injury by the fibrinolytic system. Subsequently, vascular repair is accomplished by thrombolysis and recanalization of the occluded site.

Failure of the hemostatic system to restore the integrity of an injured vessel causes bleeding. Inability to maintain the fluidity of blood results in thrombosis.

SUMMARY

- Hemostasis is a regulated process by which blood is maintained in liquid state inside the vessels.
- Five components namely: platelets, endothelial cells the coagulation system, coagulation regulatory system and fibrinolytic system interact with each other to provide hemostasis.
- Platelets are involved in both primary and secondary hemostasis and get activated through adhesion, secretion of their granule content and aggregation. Platelets adhere to the subendothelial collagen through vWF, release their granule content and use fibrinogen for their aggregation.
- Endothelial cells also play a role in the regulation of hemostasis. Normal endothelial cells are nonthrombogenic because of its antiplatelet, anticoagulant, and fibrinolytic properties.
- Majority of coagulation factors are derived from liver. Coagulation pathway may be initiated by intrinsic or extrinsic pathway and forms fibrin.
- Formation of clot is inhibited by three coagulation regulatory mechanisms namely: antithrombin, Protein C and S and tissue factor pathway inhibitor (TFPI).
- Fibrinolytic system digests thrombus and limits the clot formation. Plasmin generated from fibrinolytic system digests fibrin to form fibrin-split products. One of the split product known as D-dimer is used for the diagnosis of DIC.

SELF-ASSESSMENT EXERCISES

I. Short Notes

1. Normal hemostasis
2. Coagulation regulatory mechanism
3. Fibrinolytic system

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II. Multiple Choice Questions

1. **Following is required for platelet binding to vWF during hemostasis**
   A. Actin
   B. Thrombin
   C. Factor IX
   D. Glycoprotein Ib-IX
2. **Laboratory test that assesses the function of coagulation factors involved in intrinsic pathway is**
   A. Prothrombin time
   B. Partial thromboplastin time
   C. Hess test
   D. Bleeding time
3. **Laboratory test that assesses the function of coagulation factors involved in extrinsic pathway is**
   A. Prothrombin time
   B. Partial thromboplastin time
   C. Hess test
   D. Bleeding time
4. **All the following are endogenous anticoagulants which prevent coagulation in the vascular system EXCEPT**
   A. Antithrombin
   B. Plasminogen
   C. Proteins C and S
   D. Tissue factor pathway inhibitor
5. **The coagulation factor NOT utilized in intrinsic pathway is**
   A. VII
   B. VIII
   C. IX
   D. XI

ANSWERS

1. D **2.** B **3.** A **4.** B **5.** A

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Chapter 34

Bleeding Disorders: Vessel Wall Abnormalities

Chapter Outline

- Disorders of Hemostasis
- Bleeding Disorders Caused by Vessel Wall Abnormalities

DISORDERS OF HEMOSTASIS

Normal hemostasis requires a well controlled collaboration among the five components of hemostasis namely vascular endothelium, platelets, coagulation system, coagulation regulatory system and fibrinolytic system (Chapter 33).

Functions of hemostasis: The important functions of hemostasis are:

- To arrest bleeding at the site of injury or blood loss by forming a hemostatic plug.
- To remove the hemostatic plug when healing is complete.
- To maintain blood in a fluid state within the vascular system.

Normally, a delicate balance exists between the different hemostatic functions mentioned above. Hemostasis arrests bleeding from injured vessel and forms thrombi only when necessary. A deficiency or exaggeration of any one of the components of hemostasis may either lead to failure to restore the integrity of an injured vessel causing **bleeding** (hemorrhage) or inability to maintain the fluidity of blood causing **thrombosis**. Bleeding disorders are more common than thrombotic disorders.

Classification of Hemostatic Disorders

They are mainly classified as bleeding disorders and thrombotic disorders (Table 34.1)

Table 34.1: Classification of disorders of hemostasis

1. **Bleeding disorders**
   - ***Disorders of primary hemostasis***
     - **Vessel wall abnormalities**
       - Congenital, e.g. Ehlers-Danlos syndrome
       - Acquired, e.g. Henoch-Schönlein purpura,
     - **Platelet abnormalities**
       - Quantitative: Thrombocytopenia (e.g. ITP, drug-induced, congenital)
       - Qualitative: Platelet function disorders
         - Inherited, e.g. Glanzmann thrombasthenia, Wiskott-Aldrich syndrome, Bernard Soulier syndrome
         - Acquired, e.g. Uremia drugs

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- ***Disorders of coagulation system*** (disorders of secondary hemostasis)
  - Congenital: Hemophilia A, B; von Willebrand disease; other coagulation factor deficiencies [XI, VII, II, V, X]
  - Acquired: Vitamin K deficiency, liver disease, disseminated intravascular coagulation

2. **Thrombotic disorders**
   - Inherited
     - Deficiency of antithrombotic factors: Antithrombin III deficiency, protein C deficiency, protein S deficiency
     - Increased prothrombotic factors: Activated protein C (APC) resistance (Factor V mutation/factor V Leiden), prothrombin (G20210A mutation)
   - Acquired: Fibrinolytic system defects

Note: von Willebrand factor forms bridges between platelets and subendothelial components and its deficiency can also result in defect in the formation of a primary hemostatic plug.

Detailed classification of individual bleeding disorders is presented with concerned disorders (see Tables 34.2, 35.3, 35.4 and 36.2).

Bleeding Disorders

Extravasation of blood into the extravascular space is known as bleeding or hemorrhage.

Definition: Bleeding disorders is a general term for a group of conditions (acquired or inherited) in which there is an abnormal tendency to bleeding /hemorrhage due to failure of hemostasis.

They are also known as hemorrhagic disorder /hemorrhagic diathesis (diathesis is a Greek word for a predisposition or tendency). The bleeding may be heavy and prolonged which may occur after an insignificant injury or spontaneously.

Classification of bleeding disorders (Table 34.1): Disorders of hemostasis may be broadly classified as disorders of primary hemostasis and disorders of secondary (coagulation) hemostasis.

Terminologies Used in Bleeding Disorders (Fig. 34.1)

Petechiae

They are small (1 to 2 mm in diameter), red to purple hemorrhagic spots in the skin, mucous membranes or serosal surfaces. They result from blood leaking through intact endothelial lining of capillaries. They are most **commonly found with low platelet counts (thrombocytopenia) or defective platelet function.**

Purpura

The term purpura means purple. They are slightly larger (≥ 3 mm) than petechiae. The causes are **thrombocytopenia, increased vascular fragility and vasculitis.**

Note: Purpura may be classified as thrombocytopenic and non-thrombocytopenic (vascular) purpura.

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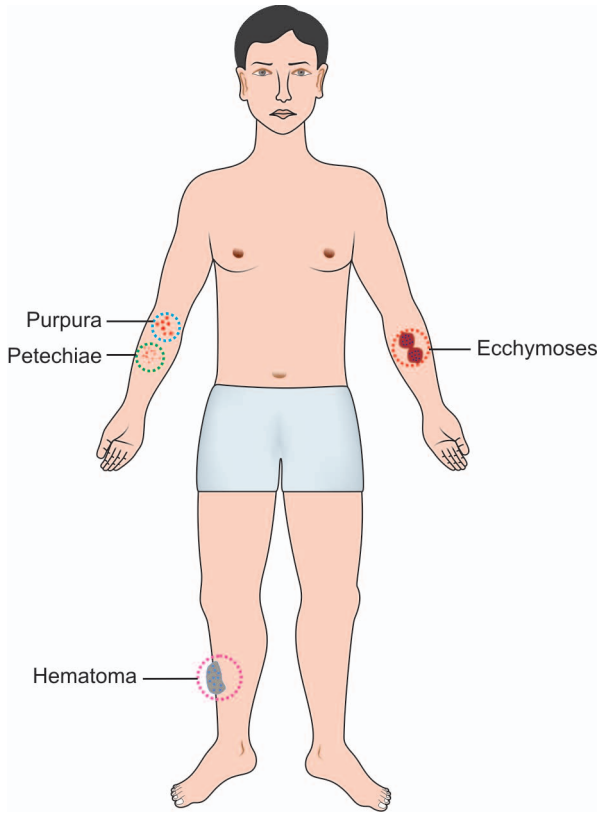


Fig. 34.1: Terminologies used in bleeding disorders

Ecchymoses

They are larger (>1 to 2 cm) and result from blood escaping through endothelium into intact subcutaneous tissue. RBCs in the lesions are degraded and the released hemoglobin gives rise to red-blue color. The pigment from heme is converted into biliverdin and then to bilirubin (blue-green color) and iron from red cells forms hemosiderin (golden-brown color). These changes are responsible for the characteristic color changes in ecchymoses.

Hematoma

It is formed when blood leaks from a vessel and collects within a tissue. It is blue or purple and slightly raised

Easy bruisability

This term is used when petechiae and ecchymoses develop with less than usual trauma.

Excess bleeding

It means bleeding occurs for a longer time and is more profuse than compared to normal.

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Epistaxis (nose bleeds): This indicates bleeding in the nasal cavity.

Depending on the site, a large accumulation of blood in a body cavity is named as hemothorax, hemoperitoneum, hemopericardium or hemarthrosis (in joints).

Thrombotic Disorders

Three primary abnormalities which cause thrombus formation (known as Virchow's triad) are: (1) endothelial injury, (2) stasis or turbulent blood flow and (3) hypercoagulability of the blood. Endothelial injury play an important role in thrombus formation in the heart or the arterial circulation. Turbulence predisposes to arterial and cardiac thrombosis whereas stasis is a major contributor to the venous thrombosis. **Hypercoagulable state** (Chapter 37) constitutes a group of inherited or acquired conditions that cause a pathologic thrombotic tendency or risk for thrombosis.

Classification of Thrombotic Disorders

Disorder of hemostasis is one of the predisposing factors that lead to hypercoagulable state. **Thrombotic disorders** due to abnormalities in hemostasis may occur due to inherited and acquired defects in two components of normal hemostasis (Chapter 33) namely: coagulation regulatory system (see Figs 33.5 and 33.7) or fibrinolytic system (removes the fibrin clot see Fig 33.6).

- Inherited causes of thrombotic disorders leading to hypercoagulable state are mainly due to defect in the coagulation regulatory system, which may result from either **deficiency of antithrombotic factors or increased prothrombotic factors** (see Table 37.1).
- Acquired or the secondary hypercoagulable states (Chapter 37) are diverse disorders that predispose patients to thrombosis by complex, multifactorial pathophysiologic mechanisms.

BLEEDING DISORDERS CAUSED BY VESSEL WALL ABNORMALITIES

The blood vessel is one of the component that is involved in hemostasis. Any structural damage to either the endothelium or the supporting collagen may result in bleeding. **Vascular purpura** is thus, a group of **disorders of blood vessels which result in bleeding**, sometimes called **nonthrombocytopenic purpura.** They are relatively common disorders and usually do not cause serious bleeding and induce small hemorrhages (petechiae and purpura) in the skin or mucous membranes. The platelet count, bleeding time and results of the coagulation tests (PT, PTT) are usually normal. Hess test (Chapter 48) is usually positive.

Classification

The bleeding disorders caused by vessel wall abnormalities are classified as inherited or acquired (Table 34.2).

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Table 34.2: Classification of bleeding disorders caused by vessel wall abnormalities

Acquired disorders

1. Due to decreased amount of connective tissue
   - Senile purpura
   - Scurvy
   - Cushing syndrome and steroid therapy
2. Due to vasculitis
   - Henoch-Schönlein purpura
   - Infections
   - Drug reactions
3. Associated with plasma cell neoplasms
   - Amyloidosis
4. Miscellaneous
   - Simple easy bruising

Inherited disorders

Hereditary hemorrhagic telangiectasia
Ehlers-Danlos syndrome
Marfan syndrome

Acquired Disorders

- *Decreased connective tissue:* Collagen is required for the support of the blood vessel wall. Any impairment in collagen formation may result in bleeding from the microvasculature.
  - **Senile Purpura:** It is age-related atrophy of supporting connective tissue of blood vessels. It is characterized by superficial, sharply demarcated, persistent purpuric spots on the forearms and other sun-exposed areas of the skin.
  - **Scurvy:** Lack of vitamin C (scurvy) is associated with microvascular bleeding resulting from impaired formation of collagens.
  - **Cushing syndrome and steroid therapy:** In **Cushing syndrome,** protein-wasting due to excessive corticosteroid production causes loss of perivascular supporting tissue.
- *Vasculitis:* Inflammation of small blood vessels is known as vasculitis and is due to the immune complexes that attach either to the endothelial cells or the subendothelial matrix.
  - **Henoch-Schönlein purpura (HSP):** It is a systemic hypersensitivity disease of unknown etiology, due to the deposition of circulating immune complexes within vessels. It is characterized by purpura, abdominal pain (due to bleeding into the gastrointestinal tract), arthralgia and acute glomerulonephritis. Microscopic examination of blood vessel shows leukocytoclastic vasculitis with perivascular infiltration of neutrophils and eosinophils.
  - **Infections:** It may produce hemorrhages in the form of petechiae and purpura, especially meningococcemia, septicemia, infective endocarditis and several of the rickettsioses. Mechanisms include invasion of vessel, septic emboli, immune complex vasculitis or result of disseminated intravascular coagulation (DIC).
  - **Drug reactions:** It may cause petechiae and purpura without thrombocytopenia. The vascular injury is mediated by drug-induced antibodies and deposition of immune complexes in the vessel walls, leading to hypersensitivity (leukocytoclastic) vasculitis.

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- *Associated with plasma cell dyscrasias*
  Amyloidosis: In systemic amyloidosis perivascular deposition of amyloid may weaken the blood vessel wall and cause purpura. This is most commonly seen in plasma cell neoplasms and presents as mucocutaneous petechiae.
- *Miscellaneous*
  Simple easy bruising: These are the purpuric lesions seen usually in women. Bruises appear spontaneously on arms and legs and resolve rapidly. Hemostatic tests are within normal range.

Inherited Disorders

Hereditary Hemorrhagic Telangiectasia (Weber-Osler-Rendu syndrome)

- It is an autosomal dominant disorder of vascular malformation characterized by dilated, tortuous, thin walled blood vessels of the skin and mucous membranes.
- They bleed easily in to the mucous membranes of the nose (epistaxis), tongue, mouth and eyes and the gastrointestinal tract. Repeated bouts of bleeding (e.g. from the gastrointestinal tract) may result in iron deficiency anemia.

Ehlers-Danlos Syndrome

- It is a congenital disorder of defective collagen synthesis characterized by joint hyper mobility skin laxity and easy bruising.
- The capillaries are poorly supported by subendothelial collagen and result in bleeding mainly in the form of ecchymoses.

SUMMARY

- Increased tendency to hemorrhage with insignificant injury are known as hemorrhagic diatheses. The cause may be due to platelet/vascular/coagulation disorders. The lesions they produce may vary from minute petechiae to large ecchymoses.
- Bleeding disorders caused by vessel wall abnormalities are relatively common disorders but usually do not cause serious bleeding and induce small hemorrhages. The cause can be inherited (Ehlers-Danlos syndrome, Weber-Osler-Rendu syndrome) or acquired (effects of aging, vitamin C deficiency, drug reactions and infections).

SELF-ASSESSMENT EXERCISES

I. Short Notes

1. Define and classify purpura
2. Henoch-Schönlein purpura

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II. Multiple Choice Questions

1. **A purple flat lesion in the skin resulting from a bleeding disorder, which is larger than 3 mm is called as**
   A. Petechiae
   B. Purpura
   C. Ecchymoses
   D. Hematoma
2. **Platelet count is NOT decreased in**
   A. Dengue fever
   B. Wiskott-Aldrich syndrome
   C. Henoch-Schönlein purpura
   D. Immune mediated lysis
3. **Henoch-Schönlein purpura is characterized by the following EXCEPT**
   A. It is a systemic hypersensitivity disease
   B. The blood vessel shows leukocytoclastic vasculitis
   C. It may be accompanied by glomerulonephritis
   D. Platelets have abnormal morphology

ANSWERS

1. B **2.** C **3.** D

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Chapter 35

Bleeding Disorders: Abnormalities of Platelet

Chapter Outline

- Introduction
- Quantitative Platelet Disorders
- Qualitative Platelet Disorders

INTRODUCTION

For effective hemostasis, an adequate number of functional platelets are required. Any decrease in platelet number (thrombocytopenia) or impairment of platelet function results in excessive bleeding following injury. Bleeding time relies on adequate platelet numbers and function rather than fibrin formation. So, it is a good guide to test the efficiency of primary hemostasis and is not usually affected by deficiencies of clotting factors.

Classification of Platelet Disorders

Platelet disorders are classified (Table 35.1) as quantitative (number of platelets) or qualitative (platelet function) in nature.

Table 35.1: Classification of platelet disorders

Quantitative platelet disorders
- Thrombocytopenia
  - Increased destruction
  - Decreased production
  - Sequestration
  - Dilutional
- Thrombocytosis

Qualitative platelet disorders
- Hereditary
  - Defective adhesion of platelets
  - Disorders of platelet secretion
  - Defective platelet aggregation
- Acquired

QUANTITATIVE PLATELET DISORDERS

Quantitative disorders of platelet may be due to decreased count (thrombocytopenia) or increased count (thrombocytosis).

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Thrombocytopenia

Definition

Decrease in the platelet count below the lower limit of 150,000/cu mm (150×10^9/L) is known as thrombocytopenia.

Clinical Features of Thrombocytopenia

Thrombocytopenia is an important cause of excessive or abnormal bleeding.

Sites of bleeding

- **Cutaneous bleeding:** It may be in the form of **pinpoint hemorrhages** (**petechiae**) and is prominent in the dependent parts where the capillary pressure is higher. It may also present with **ecchymoses** or often with a history of easy bruising and hemorrhages into soft tissues following minor trauma.
- **Mucosal bleeding:** It manifests as gum bleeding, epistaxis (nosebleeds) and hematuria. It may manifest first with melena, hematuria or excessive menstrual flow (menorrhagia).
- **Central nervous system: Intracranial bleed** (subarachnoid and intracerebral hemorrhage) are rare **serious** consequences of thrombocytopenia.

Severity of bleeding

It depends upon the platelet count (Table 35.2). Clinical symptoms of thrombocytopenia are usually not evident till the count falls below 50,000/cu mm.

Table 35.2: Clinical features associated with decreased platelet count

Platelet count/cu mm	*Clinical features*
20,000 to 50,000 (20-50 × 10^9/L)	Post-traumatic bleeding
<20.000 (<20 × 10^9/L)	Spontaneous bleeding
<10,000 (<10 × 10^9/L)	Intracranial bleeding

- When the platelet counts are in the range of **20,000 to 50,000/cu mm,** bleeding usually begins immediately after trauma (**post-traumatic bleeding**).
- **Spontaneous bleeding** usually occurs when the count falls **below 20,000/cu mm** and presents as bleeding into the skin and the mucous membranes of the gastrointestinal and genitourinary tracts.
- **Markedly decreased platelet count** (<10,000/cu mm) can cause life-threatening **intracranial bleeding**.

Bleeding due to thrombocytopenia alone is associated with a prolonged bleeding time, normal prothrombin time (PT) and activated partial thromboplastin time (APTT).

Causes of Thrombocytopenia

It can be classified into four major categories (Table 35.3).

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Table 35.3: Causes of thrombocytopenia

1. ***Increased platelet destruction***
   - **Immune mediated**
     - **Autoimmune**
       - Primary: Immune thrombocytopenic purpura (acute and chronic)
       - Secondary: Systemic lupus erythematosus, B cell lymphoid neoplasms
     - **Alloimmune**: Post-transfusion or pregnancy
     - **Drug-induced**: Quinidine, heparin, sulfa compounds
     - **Infections:** HIV infection, infectious mononucleosis, cytomegalovirus
   - **Non-immune mediated**
     - Disseminated intravascular coagulation
     - Thrombotic thrombocytopenic purpura, hemolytic uremic syndrome
     - Mechanical destruction: Prosthetic heart valves, malignant hypertension
     - Microangiopathic hemolytic anemias
2. ***Decreased production of platelets***
   - **Generalized primary diseases of bone marrow:** Aplastic anemia (congenital and acquired)
   - **Bone marrow invasion/infiltration:** Leukemia, disseminated cancer
   - **Selective impairment of platelet production**
     - Drug-induced: Alcohol, thiazides, cytotoxic drugs
     - Infections: Measles, human immunodeficiency virus (HIV)
   - **Ineffective megakaryopoiesis**
     - Megaloblastic anemia
     - Myelodysplastic syndromes
3. ***Sequestration***
   - Hypersplenism
4. ***Dilutional***

Increased platelet destruction

The most common cause of thrombocytopenia as an isolated finding (with normal hemoglobin and white cells) is increased destruction of platelets resulting in decreased platelet survival. Increased destruction of platelets may be due to an immune mediated or nonimmune mediated mechanism.

- **Immune mediated thrombocytopenia:** Immune mediated platelet destruction results secondary to circulating antiplatelet antibodies or less frequently due to attachment of immune complexes to platelets. The antiplatelet antibodies can be directed against a self-antigen on the platelets (autoantibodies) or non-self antigens (alloantibodies). Common antigenic targets of both autoantibodies and alloantibodies are the platelet membrane glycoprotein complexes IIb-IIIa and Ib-IX.
  - **Autoimmune thrombocytopenia**
    - **Primary or idiopathic thrombocytopenia:** It occurs in the absence of any known risk factors.
    - **Secondary thrombocytopenia:** Systemic lupus erythematosus
  - **Alloimmune thrombocytopenia:** Alloantibodies may develop after platelet transfusion or when platelets cross the placenta from fetus into the pregnant mother. The alloantibodies produced in the pregnant mother can cross the placenta and cause thrombocytopenia in the fetus or neonate by a mechanism similar to erythroblastosis fetalis (page 97-99).

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- **Nonimmune mediated thrombocytopenia:** Increased destruction of platelets may also occur by processes other than immune mechanisms.
  - **Disseminated intravascular coagulation (DIC):** In DIC, platelets are activated and consumed due to **activation of the coagulation system** within the blood vessels (Chapter 36).
  - **Thrombotic thrombocytopenic purpura (TTP) and hemolytic-uremic syndrome (HUS):** In these conditions, platelets are activated and consumed without activation of coagulation system.
  - **Other causes:** These include mechanical destruction of platelets seen in patients with prosthetic heart valve and diffuse narrowing of the microvasculature as in malignant hypertension.

Decreased platelet production

This group is characterized by failure of the bone marrow to deliver adequate number of platelets to the peripheral blood. It can accompany:

- **Generalized primary diseases of bone marrow:** Decreased megakaryopoiesis is seen in diseases like aplastic anemia and Fanconi anemia.
- **Bone marrow invasion/infiltration:** Marrow infiltration by leukemic blasts in AML, ALL or metastatic cancer and myelofibrosis results in replacement of hematopoietic marrow.
- **Selective suppression of the megakaryocytes:** This leads to hypoplasia of megakaryocytes in the bone marrow and may be acquired or congenital.
  - Acquired pure amegakaryocytic thrombocytopenia follows viral infections (HIV), drugs (chlorthiazides, chemotherapeutic agents and ethanol) or exposure to toxic chemicals (benzene) or radiation. Myelopoiesis and erythropoiesis are normal.
  - Congenital amegakaryocytic thrombocytopenia is a rare condition.
- **Ineffective thrombopoiesis:** Because of impaired DNA synthesis in nutritional deficiency of vitamin B_{12} or folic acid.

Sequestration/excessive pooling of platelets

- Normally, 65 to 70% of platelets are circulating and 30 to 35% are sequestered in spleen.
- **Hypersplenism:** It is a syndrome which is associated with **increased sequestration/**pooling of platelets (up to 80%) in the spleen. It can cause moderate thrombocytopenia and when necessary, hypersplenic thrombocytopenia can be ameliorated by splenectomy. Patients with marked splenomegaly, irrespective of the cause, show hypersplenism. The causes of hypersplenism include congestive splenomegaly in portal hypertension, sarcoidosis, Gaucher's and other lipid storage disorders.

Dilutional

Massive blood **transfusions** can produce dilutional thrombocytopenia. Platelets are not viable in blood stored for more than 24 hours and transfusion of which replaces only plasma volume and red cells, but not the platelets. Mild dilutional thrombocytopenia also occurs frequently during the third trimester of pregnancy.

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Idiopathic (Immune) Thrombocytopenic Purpura (ITP)

ITP is the most common form of thrombocytopenia as well as common form of immune thrombocytopenia. In ITP, there is **increased destruction of platelets** by immune mechanisms and mainly by autoimmune mechanism, hence also referred to as **autoimmune thrombocytopenia.**

Types of ITP

There are two major subtypes of primary ITP, **acute and chronic**; both are autoimmune disorders in which platelet destruction results from the formation of antiplatelet autoantibodies.

Acute immune thrombocytopenic purpura

- Acute ITP is a **self-limited** disease of **children** between 2 to 4 years and occurs with equal frequency in both sexes.
- It often presents **1 to 3 weeks after viral** (measles, rubella, EBV) **infection.**
- Platelet destruction is caused by antiplatelet autoantibodies. These are IgM type of antibodies that combine with platelets and result in destruction of platelets in the spleen.
- **Platelet count is decreased**, sometimes even below 10,000/cu mm (10×10^9/L).

Clinical Features

- The onset of thrombocytopenia is abrupt or sudden with **petechiae** over the skin, **gum bleeding, epistaxis** and mild fever which usually resolve spontaneously within 6 months. Steroid therapy is indicated only if thrombocytopenia is severe.
- Approximately 20% of the children, usually those without a viral prodrome, have persistent low platelet counts beyond 6 months similar to chronic ITP.

Chronic immune thrombocytopenic purpura

Chronic ITP is defined as persistent thrombocytopenia lasting more than 6 to 12 months. This is an indolent and more common form of primary ITP usually seen in adults.

Pathogenesis

- Chronic ITP is an **autoimmune disorder** with formation of **antiplatelet antibodies**, directed against membrane glycoproteins most often IIb-IIIa or Ib-IX of platelets. The antiplatelet antibodies can be demonstrated in approximately 80% of patients and are of the IgG type.
- The mechanism of platelet destruction is similar to that seen in autoimmune hemolytic anemias. The antiplatelet antibodies act as opsonins and are recognized by IgG Fc receptors present on mononuclear phagocytes of RE system (mainly spleen) and are destroyed there resulting in thrombocytopenia (Fig. 35.1).
- **Spleen** is not only the **major site of destruction** of platelets but also important site of autoantibody synthesis. Thus, splenectomy shows marked improvement in about 75 to 80% of patients.

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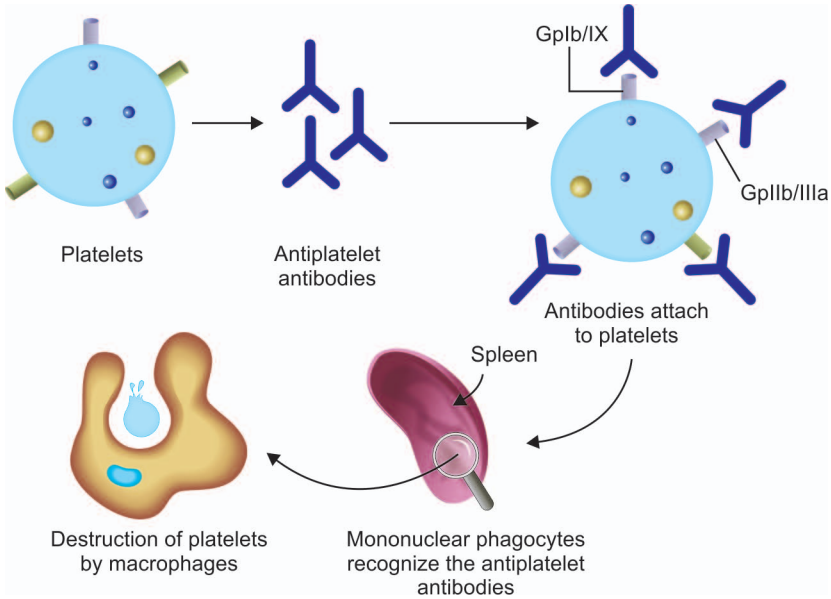


Fig. 35.1: Pathogenesis of chronic idiopathic thrombocytopenic purpura

- Although destruction of sensitized platelets is the major mechanism responsible for thrombocytopenia, the autoantibodies probably also affect production of platelets by megakaryocytes and also impair platelet function.

Clinical Features

- Chronic ITP is more common in **females** (F:M ratio is 3:1) between 20 and 40 yrs.
- Clinical features are not specific but are due to thrombocytopenia in general as mentioned in the beginning of this Chapter.
- Splenomegaly and lymphadenopathy are uncommon in primary ITP and in their presence one should consider the diagnoses other than ITP.

Laboratory Findings

Peripheral blood

- **Platelet count: Markedly reduced** and is below 80,000/cu mm (80×10^9/L).
- **Hemoglobin:** The level varies depending on the duration and amount of bleeding and ranges from 7 to 12 gm/dL.
- **Peripheral smear:**
  - **Platelets:** There is **marked reduction** in their number (thrombocytopenia). Accelerated compensatory thrombopoiesis often leads to the formation of abnormally large sized platelets (megathrombocytes/giant platelets), detected easily in a blood smear.

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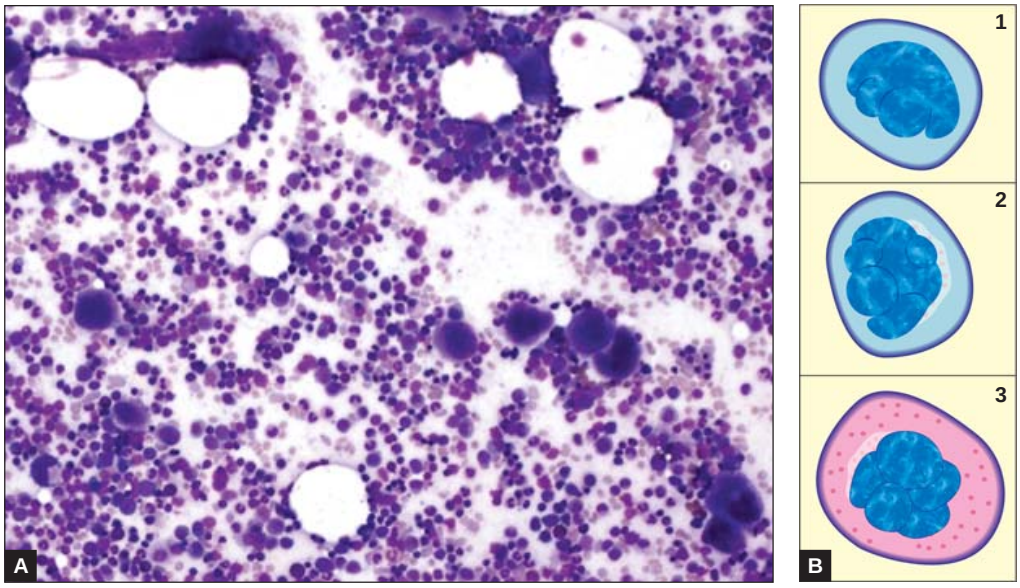
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- **RBCs:** Anemia develops in patients who have appreciable blood loss over a period of months to years. **Chronic loss** of blood may result in **microcytic hypochromic anemia**, especially in females with menorrhagia.
- **WBCs:** Usually within normal range.

Bone marrow

Bone marrow examination is important to rule out thrombocytopenias resulting from bone marrow failure. A decrease in the number of megakaryocytes argues against the diagnosis of ITP.

- **Cellularity:** Bone marrow is **hypercellular.**
- **Megakaryopoiesis:**
  - Bone marrow shows **moderate increase in number** (Fig. 35.2A) of both **immature and mature** forms of **megakaryocytes**.
  - **Immature megakaryocytes predominate.** They have **large nonlobulated single nuclei and basophilic cytoplasm (Fig. 35.2B)**.



Figs 35.2A and B: Bone marrow in ITP showing moderate increase in number of megakaryocytes: (A) Diagrammatic appearance of megakaryocytes in different stages of maturation (1-immature to 3-mature) (B)

- **Erythropoiesis:**
  - Bleeding from thrombocytopenia may result in anemia accompanied by normoblastic erythroid hyperplasia.
  - With constant bleeding, iron deficiency may result in micronormoblastic erythroid hyperplasia.

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- **Myelopoiesis**: Normal
- **Storage iron**: In patients with severe and chronic bleeding iron deficiency develops resulting in depletion of iron stores.

Bleeding time (BT): **Prolonged**, but PT and PTT are normal.

Tourniquet test: Positive

Clotting time (CT): Normal

Tests for platelet autoantibodies: May be positive.

Spleen

The size of the spleen is normal. On histological examination, spleen shows nonspecific changes like congestion of the sinusoids and prominent germinal centers. In many, scattered megakaryocytes are found within the sinuses and sinusoidal walls which may represent a mild form of extramedullary hematopoiesis due to elevated levels of thrombopoietin.

Other Causes of Immune Mediated Thrombocytopenia

- **Associated with autoimmune diseases:** Autoimmune disorders like SLE and rheumatoid arthritis may be associated with thrombocytopenia in which the platelet destruction is similar to that of ITP.
- **Drug-associated thrombocytopenia:** Many drugs can cause immune-mediated platelet destruction leading to thrombocytopenia. The most common drugs associated with thrombocytopenia are: quinine, digitoxin, quinidine, heparin, gold and sulfonamides. Chlorthiazide causes suppression of megakaryopoiesis and/or formation of antiplatelet antibodies resulting in thrombocytopenia. In chronic alcoholism, mild thrombocytopenia may develop because of suppression of megakaryopoiesis.
  Heparin-induced thrombocytopenia (HIT): Heparin is the widely used anticoagulant for prevention or treatment of thrombosis. Thrombocytopenia occurs in about 10% of patients receiving heparin and it is important to diagnose this entity to prevent its fatal consequences. Heparin can cause two clinically distinct types of syndromes:
  - **Type I:** Thrombocytopenia develops immediately following heparin therapy and is clinically insignificant with mild thrombocytopenia. It resolves after heparin is discontinued and is probably due to direct platelet-aggregation induced by heparin.
  - **Type II:** It is associated with severe thrombocytopenia and usually develops 1 to 2 weeks after starting heparin therapy (or earlier if the patient has been previously sensitized to heparin). Surprisingly, thrombocytopenia is associated with life-threatening thrombosis in both veins and arteries, known as **"white clot syndrome"**.
    Pathogenesis (Fig. 35.3): Heparin binds to platelet factor 4(PF_4) released from platelets and forms a complex (heparin/PF_4 complex) in the circulation. The antibodies formed against

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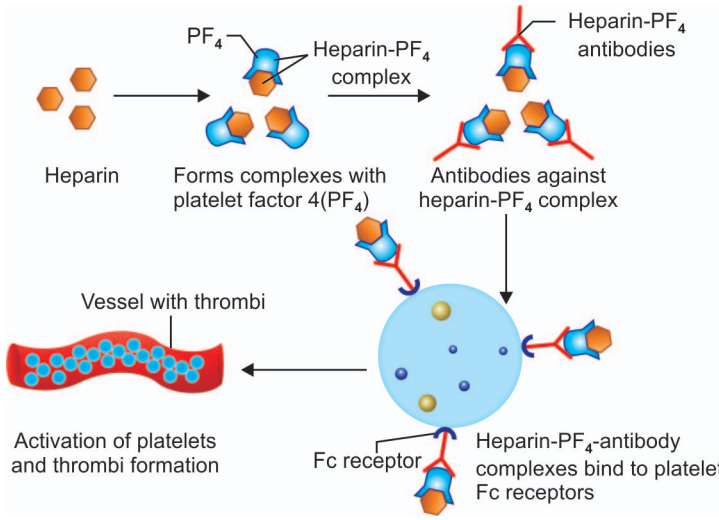


Fig. 35.3: Pathogenesis of heparin induced thrombocytopenia

these complexes activate platelets, promoting thrombosis even in the presence of marked thrombocytopenia. If heparin is not immediately discontinued, thrombosis of large arteries may lead to vascular insufficiency and emboli from deep venous thrombosis can result in fatal pulmonary thromboembolism.

- **HIV-associated thrombocytopenia:** HIV infection may result in thrombocytopenia due to both impaired production and increased destruction of platelets.
  - Like T cells, megakaryocytes also have CD4 and CXCR4, the receptor and coreceptor respectively, for HIV and are thus liable for infection by HIV. Infected megakaryocytes have **impaired platelet production** and are also susceptible to apoptosis.
  - HIV infection also stimulates B lymphocytes to produce immunoglobulins, including autoantibodies, which predispose to the **immune-mediated destruction** by the mononuclear phagocyte system.

Nonimmune Thrombocytopenia: Thrombotic Microangiopathies

Thrombotic microangiopathy is a spectrum of clinical syndromes characterized by widespread formation of **platelet-fibrin thrombi in the microcirculation**, mainly composed of platelet aggregates. Consumption of platelets causes **thrombocytopenia**; the intravascular thrombi result in **microangiopathic hemolytic anemia** and consequent organ damage. The early and timely diagnosis of thrombotic microangiopathy must be made because the delay carries the attendant risk of sudden death. Two closely related entities namely TTP and HUS are included under thrombotic microangiopathy.

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Thrombotic Thrombocytopenic Purpura (TTP) and Hemolytic-Uremic Syndrome (HUS)

The classic five symptoms of TTP are microangiopathic hemolytic anemia (MAHA), thrombocytopenia, transient neurologic symptoms, fever and renal failure. HUS is distinguished from TTP by the absence of fever and neurologic symptoms, the prominence of acute renal failure (uremia), primarily affects children and different pathogenesis. However, the clinical distinctions between them are often blurred.

Pathogenesis of TTP (Fig. 35.4)

- **Normally endothelial cells and megakaryocytes secrete** normal **vWF multimers** into the plasma. These vWF **multimers** spontaneously develop into **unusually large multimers** which are quite effective in **mediating platelet adhesion**. A plasma protease enzyme called **ADAMTS 13** ("vWF metalloprotease") **regulates** the activity of **vWF** by cleaving the hemostatically active unusually large multimers into normal multimers. Thus, ADAMTS 13 regulates the size of vWF multimers and **prevents platelet adhesion**. ADAMTS 13 is not related to tissue metalloproteases which cleave extracellular matrix.
- **TTP patients** have an inherited or acquired **deficiency of ADAMTS 13**. The deficiency leads to **accumulation** of unusually **large multimers of vWF** in plasma. These unusually large multimers may adhere to the endothelial cell surface and **promote platelet adhesion**, or circulate and promote intravascular **platelet aggregation**. Thus, spontaneous **activation of the coagulation** cascade occurs.
- This results in **platelet-fibrin thrombi throughout the microcirculation**, leading to tissue **ischemia and infarction** that are characteristic of TTP.

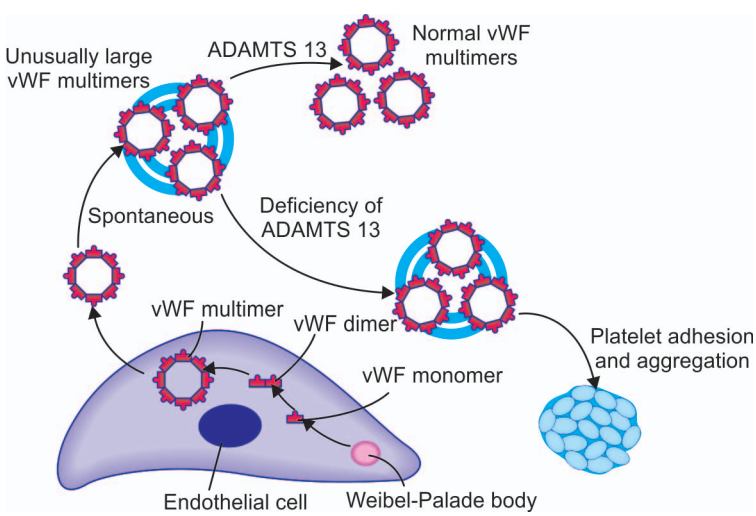


Fig. 35.4: Pathogenesis of thrombotic thrombocytopenic purpura

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Pathogenesis of HUS (Fig. 35.5)

Patients with HUS have **normal** levels of **vWF** metalloprotease, indicating a different pathogenesis than that of TTP.

- HUS develops following **damage to the endothelium** by toxins, drugs or radiation. One main cause of HUS in children and the elderly is infectious gastroenteritis caused by *Escherichia coli* strain 0157:H7. *E. coli* produces a Shiga-like toxin which is absorbed from the inflamed gastrointestinal mucosa.
- The toxin enters circulation and damages endothelial cells of microvasculature, mainly in the renal glomerular capillaries and **initiates platelet activation and thrombi formation**.

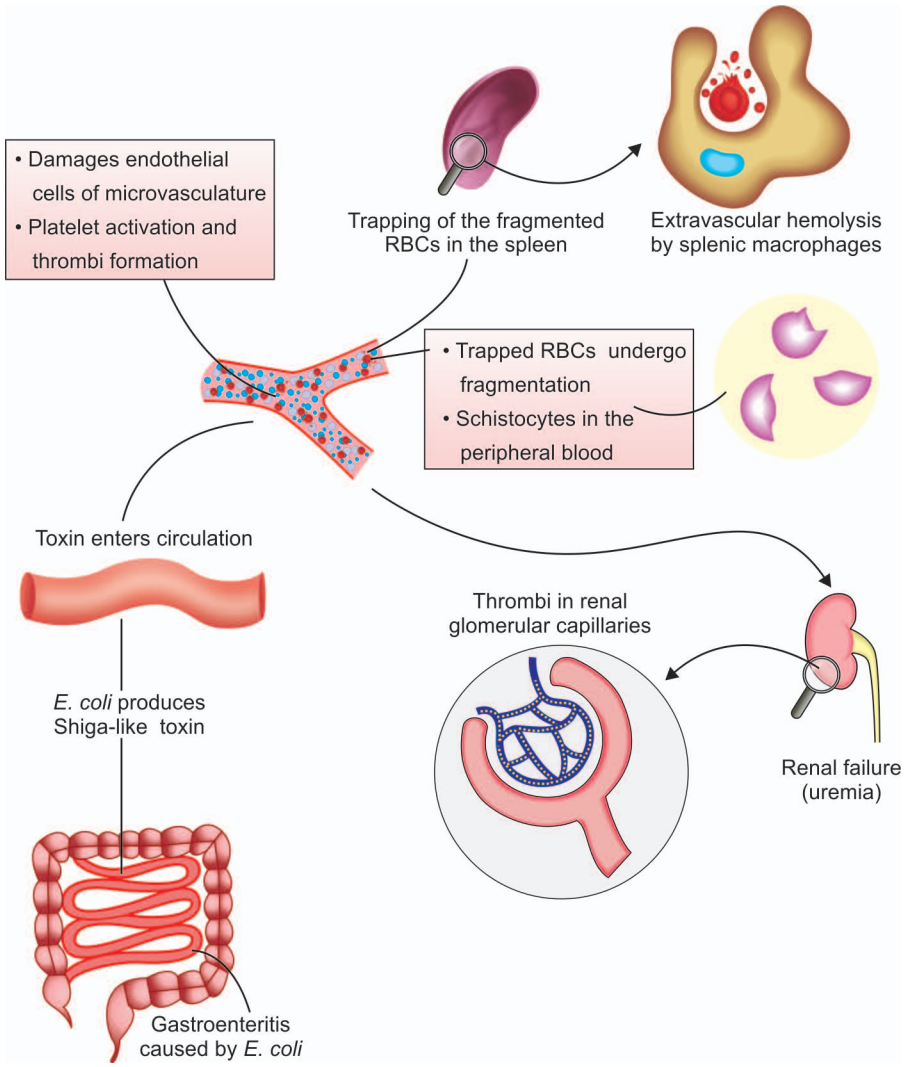


Fig. 35.5: Pathogenesis of HUS

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- **Red cells** get trapped in the formed thrombi, **undergo fragmentation** resulting in schistocytes.
- Splenic trapping of the fragmented red cells causes **extravascular hemolysis**.

Clinical features: HUS develops in children few days after a bloody diarrhea. With appropriate supportive care, they usually recover completely but in more severe cases renal damage may result in death. HUS can also develop in adults following certain drugs and radiation therapy that damage endothelial cells.

Laboratory diagnosis of thrombotic microangiopathies

Peripheral blood

- **Hemoglobin:** Decreased usually less than 6 gm/dL.
- **Platelet count:** Markedly reduced often below 20,000/cu mm (20×10^9/L).
- **Peripheral smear**
  - **RBCs:** Show fragmented red cells (schistocytes), polychromatophils, nucleated RBCs and microspherocytes.
  - **WBCs:** Show mild leukocytosis with a shift to left.
  - **Platelets:** Markedly reduced.
- **Reticulocyte count:** Increased.

Prothrombin time (PT) and activated partial thromboplastin time (APTT) are usually normal, because the coagulation system is not activated.

Urine: Shows moderate proteinuria and both gross and microscopic hematuria.

Morphology

Blood vessels: Characteristic feature is the deposition of **PAS-positive platelet-fibrin microthrombi in arterioles and capillaries** throughout the body. It is mainly seen in the heart, brain and kidneys.

Both DIC and thrombotic microangiopathies have common features like microvascular occlusion and microangiopathic hemolytic anemia, but differ in their pathogenesis. **In TTP and HUS** (unlike DIC), activation of the coagulation cascade is not a primary event, and hence **PT** and **PTT (test for coagulation)** are usually **normal**.

Thrombocytosis

Platelet count more than 4,50,000/cu mm is known as thrombocytosis (see Table 41.10).

QUALITATIVE PLATELET DISORDERS

Qualitative disorders of platelet function produce defects in the formation of hemostatic plug and thus result in bleeding. These disorders are characterized by prolonged bleeding time and normal platelet count. Qualitative defects of platelet function can be hereditary/congenital or acquired (Table 35.4).

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Table 35.4: Classification of platelet functional (qualitative) disorders

A. **Hereditary**
   1. ***Disorders of platelet adhesion***
      Bernard-Soulier syndrome
   2. ***Disorders of platelet secretion***
      Storage pool deficiency
   3. ***Disorders of platelet aggregation***
      Glanzmann thrombasthenia

B. **Acquired**
   1. ***Drugs:*** Aspirin, non-steroidal anti-inflammatory drugs (NSAIDs), dipyridamole, sulfinpyrazone
   2. ***Renal failure:*** Uremia
   3. ***Hematologic malignancies:*** Myeloproliferative neoplasms and myelodysplastic syndromes

Hereditary Disorders of Platelet Function

It can be classified into three groups on the basis of specific functional abnormality: (1) defects of adhesion, (2) disorders of platelet secretion (release reaction) and (3) defects of aggregation **(Fig. 35.6).**

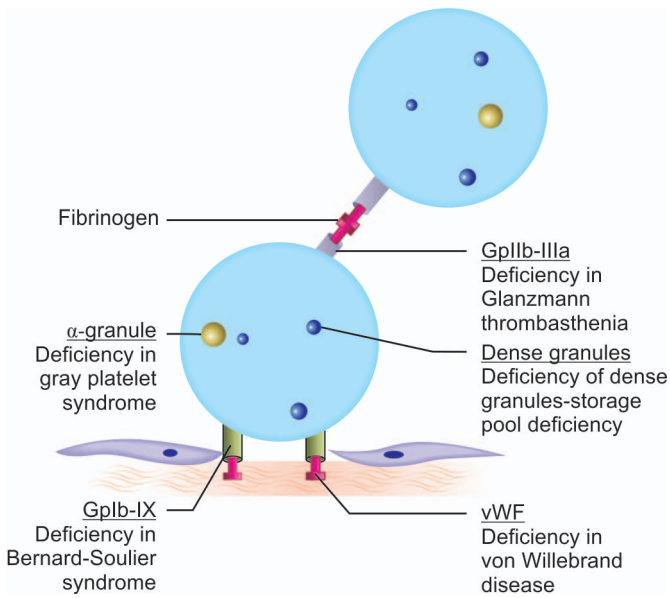


Fig. 35.6: Functional disorders of platelets

- *Defective adhesion of platelets*: Two factors are important for adhesion of platelets to subendothelial matrix during normal hemostasis. One being the platelet membrane glycoprotein complex Ib-IX, which is the receptor for the other factor, i.e. vWF.
  - **Bernard-Soulier (giant platelet) syndrome** is an autosomal recessive disorder due to a hereditary deficiency of the platelet membrane glycoprotein complex Ib-IX.

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  - Patients present with purpura, bruising, epistaxis and gingival bleeding during early life.
  - Peripheral smear examination shows mild thrombocytopenia and large platelets *(giant platelets)*.
- **von Willebrand's disease** is characterized by reduced synthesis of vWF as a result of which platelet adhesion to subendothelial collagen is defective. Since it is a plasma protein disorder rather than a platelet functional disorder, it is discussed with coagulation disorders (page no 313-314).

- ***Disorders of platelet secretion:*** Defect in the secretion may be due to lack of normal storage granules or due to defect in release of granules namely, dense granules or alpha granules.
  - **Storage pool deficiency (SPD)** is an autosomal dominant heterogeneous group of disorders in which there is a deficiency of dense granules.
  - **Gray platelet syndrome** is due to the lack of α-granule proteins because of which the platelets and megakaryocytes appear gray (pale) on peripheral blood smear.
- *Defective platelet aggregation*
  - **Glanzmann thrombasthenia** is an autosomal recessive disorder. It is due to the deficiency of glycoprotein IIb-IIIa, a protein complex that helps in the formation of "bridges" between platelets by binding fibrinogen. There is defective aggregation of platelets in response to platelet aggregators like adenosine diphosphate (ADP), collagen, epinephrine or thrombin. Clinical presentation is in the form of epistaxis and bleeding from gums.

Acquired Defects of Platelet Function

- **Drugs:** Numerous therapeutic drugs can affect platelet function, but most important are aspirin and other nonsteroidal anti-inflammatory drugs. Metabolism of arachidonic acids requires enzyme cyclooxygenase for the synthesis of platelet aggregator thromboxane A_2. Aspirin is a potent irreversible inhibitor of the enzyme cyclooxygenase and this platelet anti-aggregating effect of aspirin is the basis for its use in the prevention of thrombosis in cases of coronary artery disease or cerebrovascular stroke. The defective aggregation lasts for 7-10 days even at a low dose of 60 mg aspirin.
- **Renal failure:** Uremia produces several abnormalities in platelet function.
- **Hematologic malignancies:** In myeloproliferative neoplasms and myelodysplastic syndromes, platelet dysfunction is due to intrinsic platelet defects.

SUMMARY

- Thrombocytopenia is the most common cause of bleeding.
- Thrombocytopenia may develop due to decreased production, increased destruction or excessive pooling.
- Destruction of platelets may be due to immunological or nonimmunological mechanism.
- Acute ITP occurs commonly in children after a viral infection and they usually recover spontaneously.

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- Chronic ITP is characterized by persistent thrombocytopenia lasting for 6-12 months.
- In heparin induced thrombocytopenia, heparin forms a complex with platelet factor 4. Antibodies formed against these complexes cause destruction of platelets.
- TTP is characterized by hemolytic anemia with fragmentation of red blood cells, thrombocytopenia, fever, neurological dysfunction and progressive renal failure.
- HUS is an acquired disorder seen primarily in children and renal failure is more common than in TTP.

SELF-ASSESSMENT EXERCISES

I. Short Notes

1. Classify bleeding disorders. Describe pathogenesis and laboratory findings of idiopathic (immune) thrombocytopenic purpura
2. List the causes of thrombocytopenia
3. Describe the peripheral blood smear and bone marrow findings in ITP
4. Idiopathic (immune) thrombocytopenic purpura
5. Differences between acute and chronic ITP
6. Drug induced thrombocytopenia
7. Thrombotic thrombocytopenic purpura
8. Hereditary disorders of platelet function

II. Multiple Choice Questions

1. **Acute ITP is caused by antibodies against**
   A. Vascular endothelium
   B. Platelets
   C. Clotting factors
   D. Vessel wall
2. **In chronic ITP, the type of antibody is**
   A. IgA
   B. IgD
   C. IgG
   D. IgM
3. **The following is NOT associated with thrombocytopenia**
   A. Giant hemangioma
   B. Henoch-Schönlein Purpura
   C. Disseminated intravascular coagulation
   D. Wiskott-Aldrich syndrome
4. **The following is NOT observed in idiopathic thrombocytopenic purpura**
   A. Platelet associated IgG can be demonstrated in plasma
   B. Bone marrow shows moderate increase in number of megakaryocytes
   C. Bleeding time is prolonged
   D. Prolonged PT and PTT

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5. **All the following features are found in TTP EXCEPT**
   A. Thrombocytopenia
   B. Microangiopathic hemolytic anemia
   C. Intravascular thrombi
   D. Disseminated intravascular coagulation
6. **Bernard-Soulier syndrome is due to deficiency of**
   A. GpIb-IX
   B. GpIIb-IIIa
   C. von Willebrand's factor
   D. Factor VII
7. **Gray Platelet syndrome is due to the lack of**
   A. α-granule proteins
   B. β-granule proteins
   C. γ-granule proteins
   D. δ-granule proteins
8. **Glanzmann thrombasthenia is due to the deficiency of**
   A. Glycoprotein Ib-IX
   B. Glycoprotein IIb-IIIa
   C. von Willebrand factor
   D. Factor VII
9. **In Glanzmann thrombasthenia, the defect is in**
   A. Platelet adhesion
   B. Platelet aggregation
   C. Platelet secretion
   D. Number of platelets
10. **Hematological disorder associated with reduced platelet count is**
    A. Hemolytic anemia
    B. Acute leukemia
    C. Hemophilia
    D. von Willebrand's disease

ANSWERS

1. B **2.** C **3.** B **4.** D **5.** D **6.** A **7.** A **8.** B
9. B **10.** B

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Chapter 36

Bleeding Disorders: Abnormalities of Coagulation Factors

Chapter Outline

- Introduction
- Hereditary Deficiencies
- Acquired Coagulation Disorders
- Disseminated Intravascular Coagulation

INTRODUCTION

Bleeding disorders due to coagulation/clotting factor deficiencies present as spontaneous large ecchymoses, hematomas or prolonged bleeding following laceration or any surgical procedure. The patient typically complains of continuous oozing for days after a tooth extraction or hemarthrosis after relatively minor stress on weight-bearing knee joint. Bleeding into the gastrointestinal and urinary tracts is also common. The distinguishing features of type of bleeding between platelet/vascular disorders and coagulation disorders are given in Table 36.1.

Table 36.1: Distinguishing patterns of bleeding in platelet/vascular and coagulation disorders

Characteristics	*Platelet/vascular disorders*	*Coagulation disorders*
Onset	Spontaneous and develops immediately after trauma/surgery	Delayed bleeding after trauma/ surgery
Type of lesion	Petechiae, ecchymoses	Hematomas
Sites	Skin, mucous membrane	Deep tissues
• Mucous membrane	Common from nose, mouth, gastrointestinal and genitourinary tracts	Uncommon except from gastrointestinal or genitourinary tract
• Into the joint	Absent	Common in severe factor deficiencies
• Into the muscle	Following trauma	Spontaneous

Classification: Coagulation defect may be due to **hereditary or acquired** (Table 36.2) deficiencies of coagulation factor and it may be **quantitative or a qualitative defect.**

Table 36.2: Coagulation disorders

A. Hereditary coagulation disorders
1. von Willebrand disease
2. Hemophilia A
3. Hemophilia B
4. Others

B. Acquired (secondary) coagulation disorders
1. Vitamin K deficiency
2. Liver disease
3. Others

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HEREDITARY DEFICIENCIES

Deficiency usually involves a single clotting factor and any clotting factor except factor XII may cause a bleeding disorder. The three common hereditary disorders are:

- Hemophilia A (deficiency of factor VIII)
- Hemophilia B (deficiency of factor IX)
- von Willebrand disease (deficiency of vWF)

Note: Deficiency of factor XI is also known as Hemophilia C.

Deficiencies of Factor VIII-vWF Complex

Hemophilia A and von Willebrand disease, two of the most common inherited disorders of bleeding are caused by qualitative or quantitative defects involving the factor VIII-vWF complex.

- **Factor VIII procoagulant protein** is a co-factor required for the activation of factor X and generation of fibrin in the intrinsic coagulation pathway. In the plasma, it **binds with** a carrier protein-**von Willebrand factor** and forms a plasma factor **VIII-vWF complex.** Both components of the factor VIII-vWF complex are coded by different genes and produced by different cells. Factor VIII is synthesized predominantly in the liver (endothelial and kupffer cells) and kidney (tubular epithelial cells).
- **vWF protects factor VIII** and is important for its stability. In the circulation, vWF exists in the form of very high-molecular-weight multimers. Absence of vWF impairs the activity of factor VIII. vWF is an adhesive protein produced by vascular endothelial cells and α-granules of platelets. Its major source is endothelial cells, where it is stored in the Weibel-Palade bodies present in their cytoplasm. Depending on the location of vWF, it can be subdivided into sub endothelial and plasma vWF. It has no role in the coagulation cascade, but in primary hemostasis, vWF is the most important co-factor for adhesion of platelets to the exposed subendothelial collagen matrix by GpIb-IX. The adhesion of platelets to subendothelium by vWF is accomplished by two ways.
  - **Subendothelial vWF:** Part of vWF secreted by endothelial cells is normally deposited in the subendothelial matrix, and when the endothelium is disrupted, it **promotes platelet adhesion.**
  - **Plasma vWF:** Endothelial cells and platelets also release vWF into the circulation. When there is vascular endothelial injury, the plasma vWF also gets **adsorbed to exposed subendothelial matrix** in addition to the already deposited subendothelial vWF and further **augments adhesion of platelets.**

von Willebrand Disease (vWD)

von Willebrand disease is the most common **inherited** group of bleeding disorders with a variable clinical picture and modes of inheritance. In most cases, it is transmitted as an autosomal dominant disorder. More than 20 variants of von Willebrand disease have been described. They are grouped into two major categories:

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- **Quantitative deficiency of vWF:** Type 1 and type 3 von Willebrand diseases are associated with a decreased quantity of circulating vWF.
  - Type 1 is an autosomal dominant, relatively mild disorder and constitutes about 75 percent of all cases.
  - Type 3 is an autosomal recessive disorder. It is the least common type and is a severe disorder associated with extremely low levels of functional vWF.
- **Qualitative defects in vWF:** Type 2 von Willebrand disease accounts for 25 percent of all cases and is characterized by qualitative defects in vWF. It is inherited as an autosomal dominant disorder. There are several subtypes:
  - Type 2a is the most common of Type 2, in which vWF is expressed in normal amounts, but has defective assembly of multimers.
  - Type 2b is caused by synthesis of an abnormal vWF with increased affinity for platelets which results in thrombocytopenia.

Clinical Features

In most of the cases, the bleeding manifestations are mild and remain undetected. The common symptoms are spontaneous bleeding from mucous membranes (e.g. epistaxis), excessive bleeding from wounds or menorrhagia. In severe cases, manifestations may be similar to hemophilia A.

Laboratory Findings

- **Platelet count:** Normal.
- **Bleeding time: Prolonged** bleeding time despite a normal platelet count.
- **Clotting time:** Prolonged.
- **Tourniquet test (Hess test):** This is **positive** due to defect in platelet adhesion.
- **APTT:** Normally, vWF stabilizes factor VIII by binding to it. A deficiency of vWF gives rise to a secondary decrease in factor VIII levels and **prolonged APTT.**
- **vWF assay:** The plasma level of active vWF is **decreased**.
- **Platelet function test**: Function of vWF is assessed by **ristocetin aggregation test**. Ristocetin induces multivalent vWF multimers to bind platelet glycoprotein Ib-IX and results in bridging of platelets to each other. The resultant clumping (aggregation) of platelets is measured by aggregometer. The degree of ristocetin-dependent platelet aggregation is a measure of vWF activity. Defective ristocetin induced platelet aggregation is diagnostic of vWF.

Hemophilia

Hemophilia A and B are similar in both clinical and pathological features, the difference being in the deficient factor. Both are sex-linked recessive disorders resulting in inherited deficiency of the clotting factor or synthesis of a defective clotting factor. Males are affected and females are carriers.

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Hemophilia A (factor VIII deficiency)

- Hemophilia A is the most **common hereditary X- linked** recessive disease with a reduction in the amount or activity of **factor VIII.** About 30% of hemophiliacs have no family history and may be due to acquired mutations.
- Factor VIII serves as a cofactor for factor IX in the activation of factor X in the coagulation cascade (Fig. 33.2). Reduced amount or activity of factor VIII is associated with **life-threatening bleeding.** Bleeding is due to both inadequate coagulation and inappropriate clot removal (fibrinolysis).

Mode of Inheritance

Hemophilia is transmitted as sex-linked recessive disease and the genes for factor VIII are located on the long arm of the X-chromosome. Hemophilia does not clinically manifest when there is a normal copy of X-chromosome. **Males** with a defective/mutant factor VIII gene (hemophiliac gene) on their single X chromosome (X_H) **suffer** from hemophilia. Heterozygous females are carriers and do not express the full clinical disease because of the paired normal X-chromosome. However females with two copies of the defective X_H chromosome may rarely suffer from hemophilia.

Risks of transmission to children:

- ***When female is a carrier and male is normal (carrier female and normal male):*** The 25% of children may be normal male, 25% normal female, 25% female carrier and 25% may be hemophiliac male (Fig. 36.1).

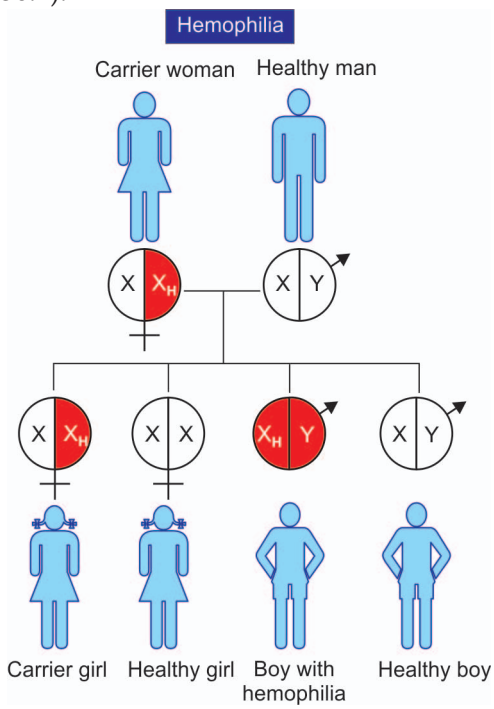


Fig. 36.1: Mode of inheritance in hemophilia

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- ***With normal female and the hemophiliac male (normal female and hemophiliac male):***
  - Hemophiliac male does not transmit the disorder to his sons, since he donates only a normal Y chromosome to his son and not the defective X_H chromosome.
  - A hemophiliac male always passes on his abnormal X_H chromosome to all his daughters and thus all daughters will be asymptomatic carriers.
- ***When female is a carrier*** (asymptomatic, heterozygous) ***and male is hemophiliac (carrier female and hemophiliac male):*** There are chances of 25% of children may be female carrier, 25% hemophiliac females, 25% normal male and 25% hemophiliac male.
- ***When female is hemophiliac and male is normal (hemophiliac female and normal male):*** The 50% children will be female carriers and 50% may be male with hemophilia.

Note: Female hemophiliac usually do not survive beyond puberty.

Molecular Genetics

Molecular genetics have detected a variety of defects in hemophilia. The causative mutations include deletions, inversions, point mutations and insertions.

Clinical Features

Clinical severity **depends on the level of Factor VIII activity with normal range expressed as 50-200%** (Table 36.3).

- Moderate to severe deficiency of factor VIII presents with easy bruising and massive bleeding following trauma or operative procedures.
- Frequent and spontaneous hemorrhages occur into the joints and are known as hemarthrosis.
- Recurrent bleeding into the joints will lead to crippling deformities.
- Patient may also present with spontaneous or post-traumatic bleeding into soft tissues. Petechiae observed in platelet and vascular disorders are not seen in hemophilia.

Table 36.3: Factor VIII level and clinical severity in hemophilia A

Clinical severity	*Level of factor VIII activity in percentage*	*Clinical features*
Mild	More than 6	In the mildest form it may be unnoticed. Bleeding develops after trauma only
Moderate	2-5	Bleeding after trauma, including dental and other surgical trauma. Easy bruising
Severe	Less than 1	Frequent and spontaneous hemorrhage into joints (hemarthrosis) and soft tissues from birth

Normal range for factor VIII: 45-158 iu/dL

Laboratory Findings

- **Bleeding time: Normal.**
- **Clotting time: Prolonged.**
- **Platelet count: Normal.**
- **Prothrombin time: Normal.**
- **Activated partial thromboplastin time (APTT): Increased** (normal 30-40 seconds) Prolongation of APTT is dependent upon the severity of deficiency of factor VIII.

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- **Factor VIII assay:** This is essential for the diagnosis, to assess the factor VIII levels and severity of disease. Factor VIII function is assessed by conducting coagulation assays with mixtures of patient plasma and factor VIII-deficient plasma.

Carrier detection: DNA markers are used as screening tests to detect female carriers and prenatal diagnosis of affected fetuses.

Complications of Hemophilia

- **Deforming arthritis and contractures:** The most frequent complication is deforming arthritis due to repeated bleeding into the joints. Organization and fibrosis of intramuscular hematomas results in contractures of involved muscles.
- **Anemia:** Excessive, spontaneous or repeated bleeding may result in anemia.

Causes of Death

- **Intracranial hemorrhage:** Severe deficiency of factor VIII may result in spontaneous, fatal intracranial hemorrhage.
- **Prolonged bleeding:** Rarely, prolonged bleeding following surgical procedures may be fatal.

Treatment

Presently available factor VIII concentrates are free of hepatitis viruses and HIV. Recombinant factor VIII is available and is replacing the therapy with factor VIII concentrate. Gene therapy is also under trial and should be weighed against the potential risks associated with it.

Complications of Therapy

Former replacement therapy with clotting factor concentrates prepared from pooled human plasma has led to other diseases in patients receiving such treatment.

- **Hepatitis:** Viral hepatitis was universal in hemophiliacs treated with concentrates prior to the introduction of virucidal preparation using heat and solvents. Hepatitis may progress to cirrhosis and liver failure leading to death.
- **AIDS:** This used to be one of the complications in patients who received fresh frozen plasma (FFP) or cryoprecipitate, when screening tests for HIV were not available.
- **Factor VIII inhibitors:** About 15% of the patients receiving factor VIII therapy develop inhibitory antibodies that bind and inhibit factor VIII. The infused clotting factor is rendered ineffective by these inhibitors and makes further management difficult.

Hemophilia B (Christmas Disease, Factor IX Deficiency)

Both factor VIII and IX together activate factor X in coagulation cascade. Thus, severe factor IX deficiency is **clinically indistinguishable from hemophilia A.** It is also inherited as an X-linked recessive trait and presents with variable clinical severity. Assay of factor IX should be done to diagnose Christmas disease (named after the first patient). Recombinant factor IX is used for treatment.

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Clinical Features

Clinical features are usually milder than those of hemophilia A. In both the diseases, hemarthrosis is the common presentation. Treatment is by infusion of purified or recombinant factor IX.

Laboratory Findings

Similar to hemophilia A

- **Bleeding time:** Normal
- **Clotting time: Prolonged**
- **Platelet count:** Normal.
- **Prothrombin time:** Normal.
- **Activated Partial Thromboplastin Time (APTT):** Increased (normal 30-40 seconds).
- **Factor IX assay:** Factor IX is decreased.

Other Hereditary Coagulation Disorders

The other coagulation disorders namely afibrinogenemia, dysfibrinogenemia and deficiency of factors like XIII, X and VII are rare.

Laboratory tests in hereditary disorders are summarized in Table 36.4.

Table 36.4: Summary of laboratory test in hereditary coagulation disorders

	Hemophilia A	*Hemophilia B*	*von Willebrand disease*
Bleeding time	N	N	Increased
Prothrombin time	N	N	N
APTT	Increased	Increased	Increased
Thrombin time	N	N	N
Factor VIII	Decreased	N	Low or Normal
Factor IX	N	Decreased	N
vWF	N	N	Decreased
Platelet count	N	N	N

ACQUIRED COAGULATION DISORDERS

Coagulation Factor Abnormalities

The majorities of coagulation disorders are acquired and are usually characterized by multiple clotting abnormalities.

- **Vitamin K deficiency**
  - Vitamin K is a fat-soluble vitamin and requires bile for its absorption. Vitamin K is required by liver for the production of factors II, VII, IX, X, protein C and protein S.
  - Deficiency of vitamin K results in release of the above coagulation factors in incomplete and inactive form from the liver. Vitamin K deficiency may develop when absorption is defective as in obstructive jaundice, pancreatic disease or small bowel disease.

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- In neonates, vitamin K levels are low and are due to lack of gut bacteria and low concentrations of the vitamin in breast milk. Liver immaturity along with low level of vitamin K may produce life-threatening hemorrhage during the first week of life known as ***hemorrhagic disease of the newborn.***
- The bleeding manifestations show a dramatic response to parenteral vitamin K therapy.
- The oral anticoagulant drug warfarin is a vitamin K antagonist. It inhibits the synthesis of coagulation factors II, VII, IX and X and is associated with a risk of hemorrhage.

- **Liver disease**
  - Liver synthesizes virtually all the clotting factors and severe liver disease is associated with a hemorrhagic diathesis.
  - Liver disease if associated with hypersplenism may cause thrombocytopenia and a qualitative platelet disorder. Massive fatal hemorrhage may develop especially from esophageal varices (one of the complications of cirrhosis).
- **Other causes:** Disseminated intravascular coagulation which involves deficiency of several coagulation factors.

Inhibitors of Coagulation Factors

Acquired inhibitors of coagulation factors (circulating anticoagulants) are usually autoantibodies. Most of these circulating anticoagulants are against factor VIII and vWF. In hereditary coagulation disorders, therapeutic administration of plasma concentrates containing the deficient factor, especially hemophilia, may stimulate production of circulating anticoagulants. The anticoagulants can also develop in patients with autoimmune disorders like systemic lupus erythematosus and rheumatoid arthritis. Bleeding is uncommon, but these patients have a tendency for thrombosis.

DISSEMINATED INTRAVASCULAR COAGULATION

Definition: Disseminated intravascular coagulation (DIC) is a widespread thrombohemorrhagic disorder in which a **combination of thrombosis and hemorrhage** develops as a secondary complication of wide variety of disorders.

Etiology and Pathogenesis (Fig. 36.2)

DIC is not a primary disease but develops as a process secondary to several diverse diseases. DIC could result from pathologic activation of the extrinsic and/or intrinsic pathways of coagulation or the impairment of clot-inhibiting mechanisms. Even though in acute DIC, the clinical manifestation is widespread hemorrhage, it is primarily a thrombotic/coagulative disorder.

Mechanism of Thrombi/Clot Formation

Two major mechanisms that initiate the thrombotic process of DIC are:

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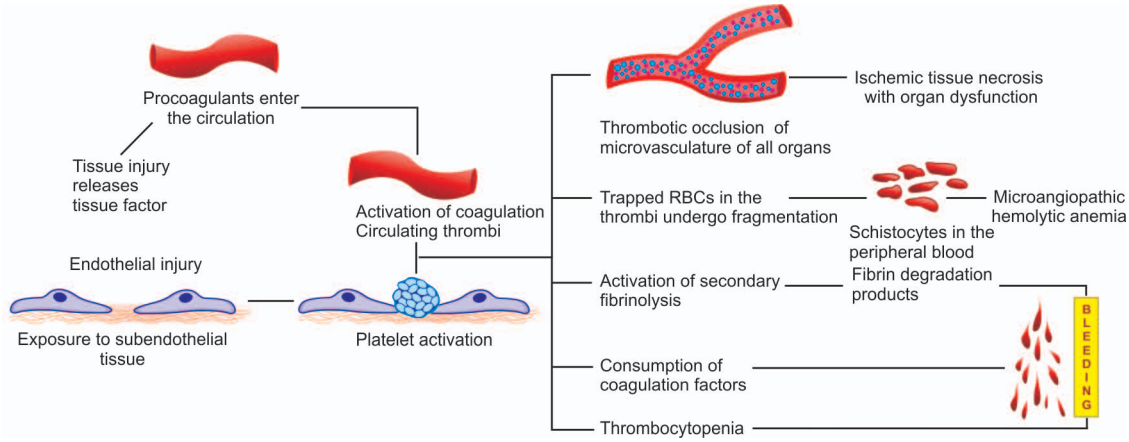


Fig. 36.2: Pathogenesis of thrombosis, ischemic tissue necrosis and bleeding in disseminated intravascular coagulation

- Entry of **procoagulant (thromboplastic) substances** into the circulation.
- Widespread **endothelial injury.**

Entry of procoagulant (thromboplastic) substances into the circulation

- The basic process starts with the **entry of procoagulant** substances into the circulation. The source of procoagulant substance in majority of cases is tissue factor. The placenta and uterine contents are rich sources of tissue factor.
- Tissue factor gains access to blood by tissue injury. Tissue injury may be induced by tissue destruction due to trauma, burns, surgery, sepsis, malignancy.
- In sepsis, TNF induces endothelial cells to express tissue factor on their cell surfaces. In some, the source of procoagulants may be other than tissue factor. The examples include mucus (released from certain adenocarcinomas) and cytoplasmic granules (e.g. acute promyelocytic leukemia cells).
- **Tissue factor activates the coagulation system** and results in widespread (disseminated) deposition of fibrin-platelet thrombi.

Widespread endothelial injury

- Endothelial injuries expose the thrombogenic subendothelial matrix leading to **activation of both platelets and coagulation pathway** and thrombi formation.
- In sepsis, TNF promotes the adhesion of leukocytes to endothelial cells and reactive oxygen species and preformed proteases from leukocytes can damage endothelial cells.
- Widespread endothelial injury may also result from deposition of immune complexes (e.g. SLE), temperature extremes (e.g. burns, heat stroke), or microbial infection (e.g. meningococci).

Consequences of thrombi formation: Microvasculature consists of arterioles, capillaries and venules throughout the body. Both procoagulant substances and endothelial injury activate coagulation resulting in fibrin-platelet **thrombi formation** (clot) in the microvasculature. In the

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process, clotting factors, fibrin and platelets are consumed. Hence, it is also referred to as **consumptive coagulopathy** or **defibrination syndrome.** In acute **DIC**, coagulation factors and platelets are consumed in excess rate than the synthetic capacity of liver and bone marrow megakaryocytes respectively. Widespread deposition of fibrin-thrombi within the microcirculation leads to:

- **Ischemic necrosis:** Though thrombi are found in the microvasculature of any organ, majority are seen in the brain, heart, lungs and kidney. Microvascular obstruction leads to **widespread ischemic changes** with **micro-infarcts or large areas of infarction.** Multiorgan failure develops as a result of ischemic necrosis of vulnerable organs. These organs are also sites of bleeding which may be fatal when it occurs in the brain and gut.
- **Microangiopathic hemolytic anemia:** Red blood cells trapped in the intravascular fibrin-thrombi deposits undergo fragmentation as they squeeze through the narrowed microvasculature. These red blood cells appear as schistocytes in blood smears; but, frank hemolytic anemia is unusual in DIC.

Mechanism of hemorrhagic diathesis: The fibrin-thrombi formed activate secondary fibrinolytic system and plasmin is generated. The plasmin cleaves/digests fibrinogen and fibrin and generates split products [FSPs or fibrin degradation products (FDP)]. FDPs themselves have potent anti-coagulant and anti-platelet effect and contribute to the **hemorrhagic diathesis**. Thus, the bleeding diathesis in DIC are due to:

- Consumption of platelets
- Consumption of coagulation factors
- Activation of fibrinolytic system.

Major Disorders Associated with DIC (Table 36.5)

DIC always has an underlying cause or disorder and triggering conditions are often multiple and many of these are associated with an unfavorable outcome.

- ***Infection:*** It is the most common cause of **DIC** and is particularly associated with gram-negative or gram-positive sepsis. Infection may directly or indirectly activate platelets through release of toxins, TNF or immune complex formation.
- ***Obstetric complications:*** The placenta and uterine contents are rich sources of tissue factor and other procoagulants. In amniotic fluid embolism, patient develops acute fulminant and often fatal DIC, whereas with a retained dead fetus it is of subacute or chronic type. Other obstetric conditions associated with DIC include abruptio placentae, toxemia and septic abortion.
- ***Neoplasms***: Chronic forms of DIC may develop in a variety of malignant neoplasms, particularly carcinoma of pancreas, prostate, lung and mucin-secreting adenocarcinoma of stomach, in which thrombotic rather than bleeding manifestations predominate.
- ***Massive tissue injury:*** In tissue injury caused by trauma, surgery and shock, the development of DIC is related to the extent of tissue damage and the organs involved. Brain is a rich source of tissue factor, so traumatic brain injury can cause acute **DIC.** In massive tissue injury, the major triggering factor is the release of procoagulant tissue factor.

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Table 36.5: Major disorders associated with disseminated intravascular coagulation

Infections
- Gram-negative bacterial sepsis
- Meningococcemia and other bacteria
- Fungi, viruses, Rocky Mountain spotted fever, malaria

Obstetric Complications
- Retained dead fetus
- Septic abortion
- Abruptio placentae
- Amniotic fluid embolism
- Toxemia and pre-eclampsia

Neoplasms
- Carcinomas of pancreas, prostate, lung and stomach
- Acute promyelocytic leukemia

Massive Tissue Injury
- Traumatic
- Burns
- Fat embolism
- Surgery

Vascular disorders
- Aortic aneurysm, giant hemangioma

Immunologic reactions
- Transfusion reactions
- Transplant rejection

Respiratory distress syndrome

Miscellaneous
- Snakebite, liver disease, acute intravascular hemolysis, shock, heat stroke, hypersensitivity, vasculitis

- *Vascular disorders:* In aortic aneurysms, giant hemangiomas and other vascular malformations, **DIC** is initiated locally in the abnormal vasculature but can spread into the systemic circulation.
- *Miscellaneous*
  - **Snake bite:** Snake bites can induce **DIC** by introduction of exogenous toxins and release of endogenous tissue factor via tissue necrosis. Snake venom contains many substances that can affect coagulation and endothelial permeability.
  - **Liver disease:** The exact mechanism of **DIC** in liver disease is not known. Impaired clearance of activated clotting factors, plasmin and FDP may possibly exacerbate intravascular coagulation.

Clinical Features

Disseminated intravascular coagulation (DIC) is a serious, often fatal, clinical condition which needs an immediate diagnosis and management. The symptoms of DIC depend on the nature, intensity and duration of the underlying disorder.

- Depending on the onset, DIC is divided into *acute, subacute or chronic forms.*
- Low-grade **DIC** may be asymptomatic and can only be diagnosed by laboratory findings.
- DIC can present with signs and symptoms related to tissue hypoxia and infarction caused by the microvascular thrombosis; or with bleeding diathesis due to depletion of factors and activation of fibrinolytic mechanisms or both (Table 36.6).

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Table 36.6: Effects and signs of DIC

Organ/site	*Due to thrombi in microvasculature*	*Due to hemorrhagic diathesis*
Central nervous system	Multifocal, delirium, coma	Intracerebral bleeding
Renal system	Cortical necrosis-oliguria, azotemia	Hematuria
Skin	Focal ischemic necrosis, gangrene	Petechiae, ecchymoses, bleeding at the sites of venipuncture
Gastrointestinal tract	Acute ulceration	Massive bleeding
Respiratory tract	Acute respiratory distress syndrome	
Mucous membranes	Epistaxis, gingival bleeding	
Peripheral circulation	Fragmentation of trapped RBCs-microangiopathic hemolytic anemia	

- **Bleeding** is the most common clinical feature in acute **DIC.** It may manifest as ecchymoses, petechiae or bleeding from mucous membranes or at the sites of venipuncture.
- **Microvascular thrombi** cause ischemic necrosis of the organ with resultant dysfunction of the involved organ. Organ dysfunction may manifest as hepatic, renal, cardiac or respiratory failure along with neurological disturbances. It may also result in gangrene of extremities and hemorrhagic necrosis of the skin.

Two clinical manifestations of DIC as a result of involvement of the endocrine glands are:

- **Waterhouse-Friderichsen syndrome:** It develops in meningococcemia and is characterized by massive adrenal hemorrhages due to fibrin thrombi within the adrenal cortex.
- **Sheehan postpartum pituitary necrosis:** This complicates labor and delivery and is a form of DIC.

The prognosis depends on the underlying disorder. Treatment is largely removal of the underlying cause and replacement of clotting factors and platelets. Mortality is high in severe cases.

Laboratory Findings in DIC

- *Screening assays*
  - **Platelet count:** Decreased due to utilization of platelets in microthrombi.
  - **Prothrombin time:** Increased.
  - **APTT:** Increased as a result of consumption and inhibition of the function of clotting factors.
  - **Thrombin time (TT):** Increased because of decreased fibrinogen.
  - **Fibrinogen:** Decreased.
  - **Peripheral smear:** Microangiopathic hemolytic anemia
- *Confirmatory tests*
  - **FDP** (fibrin degradation/split products): Secondary fibrinolysis results in generation of FDPs, which can be measured by latex agglutination.
  - **D-dimer test:** It is specific for diagnosing DIC.

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SUMMARY

- Bleeding disorders due to deficiency of coagulation factor can be hereditary or acquired.
- Hemophilia A, hemophilia B and von Willebrand disease are the three important hereditary coagulation disorders.
- Hemophilia A is due to deficiency of factor VIII and hemophilia B due to factor IX deficiency.
- Qualitative or quantitative defects in vWF cause vWD. The diagnosis and classification of its subtypes requires laboratory assays.
- Acquired causes of coagulation disorders include liver disorder, vitamin K deficiency and DIC.
- DIC is a widespread disorder in which both thrombosis and hemorrhage develop as a secondary complication of wide variety of disorders.

SELF-ASSESSMENT EXERCISES

I. Short Notes

1. Hemophilia
2. Laboratory diagnosis of hemophilia
3. Differences between hemophilia and von Willebrand disease
4. von Willebrand disease
5. Laboratory diagnosis of DIC
6. Enumerate the causes and laboratory diagnosis of DIC
7. Disseminated intravascular coagulation

II. Multiple Choice Questions

1. **The most common mode of inheritance of von Willebrand disease is**
   A. Autosomal recessive
   B. Autosomal dominant
   C. X-linked dominant
   D. X-linked recessive
2. **The following is TRUE with regards to von Willebrand disease**
   A. Normal BT
   B. Increased FDP
   C. Prolonged APTT
   D. Decreased platelet count
3. **The laboratory test useful for assessing the function of vWF is**
   A. Ristocetin aggregation test
   B. Platelet aggregation test
   C. Prothrombin time
   D. Clotting time
4. **The mode of inheritance of hemophilia A is**
   A. Autosomal dominant
   B. Autosomal recessive
   C. X-linked recessive
   D. X-linked dominant

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5. **Hemophilia A is due to the deficiency of**
   A. Factor V
   B. Factor VIII
   C. Factor IX
   D. von Willebrand factor
6. **Bleeding manifests in hemophilia, when the level of factor VIII activity is**
   A. 60 to 75%
   B. 40 to 60%
   C. 25 to 40%
   D. Below 25%
7. **All of the following laboratory findings are true in hemophilia, EXCEPT**
   A. Bleeding time is prolonged
   B. Clotting time is prolonged
   C. Platelet count is normal
   D. Prothrombin time is normal
8. **Christmas disease is due to deficiency of factor**
   A. VIII
   B. IX
   C. X
   D. XI
9. **DIC is seen in the following conditions EXCEPT**
   A. Acute promyelocytic leukemia (M3)
   B. Fat embolism
   C. Amniotic fluid embolism
   D. Chronic myeloid leukemia
10. **The following carcinomas may be associated with DIC EXCEPT**
    A. Stomach
    B. Lung
    C. Prostate
    D. Penis
11. **The specific diagnostic test for disseminated intravascular coagulation is**
    A. D-dimer assay
    B. Prothrombin time
    C. Assay of factor IX
    D. Activated partial thromboplastin time
12. **All of the following are laboratory findings in DIC EXCEPT**
    A. Increased prothrombin time
    B. Increased fibrinogen in the serum
    C. Increased partial thromboplastin time
    D. Increased fibrin degradation products in the blood

ANSWERS

1. B **2.** C **3.** A **4.** C **5.** B **6.** D **7.** A **8.** B
9. D **10.** D **11.** A **12.** B

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CHAPTER 37

Thrombotic Disorders: Hypercoagulable States

CHAPTER OUTLINE

- Introduction
- Inherited Hypercoagulable States
- Acquired Hypercoagulable States

INTRODUCTION

Endothelial injury, stasis or turbulent blood flow and hypercoagulable states of the blood (Virchow's triad) are the three important contributing factors that result in thrombosis. Hypercoagulable state is a less frequent contributor to thrombotic states, which may result (Table 37.1) from a primary (inherited disorders of hemostasis) or secondary to a hematological disorder (e.g. myeloproliferative neoplasms, PNH)/non-hematological condition (other secondary/acquired causes).

Definition: Hypercoagulable state (also known as thrombophilia) is defined as a group of inherited or acquired conditions that are associated with increased tendency or risk to develop thrombosis.

Causes: Hypercoagulability state can be *primary* (inherited/congenital/genetic) or *secondary* (acquired) (Table 37.1).

Table 37.1: Major causes of hypercoagulable state

A. Inherited (congenital/primary)

Deficiency of antithrombotic (anticoagulant) factors

1. Antithrombin III deficiency
2. Protein C deficiency
3. Protein S deficiency

Increased prothrombotic factors

1. Activated protein C (APC) resistance (Factor V mutation/ factor Va/ factor V Leiden)
2. Excessive levels of prothrombin (prothrombin G20210A mutation)
3. High levels of factors VII, XI, IX, VIII; von Willebrand factor; fibrinogen

B. Secondary (acquired)

1. Antiphospholipid antibody syndrome
2. Venous stasis
   - Prolonged immobilization
   - Congestive cardiac failure
   - Prolonged bed rest

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3. Increased platelet activation:
   - Hematological disorders: Myeloproliferative neoplasms (polycythemia vera, essential thrombocythemia, myelofibrosis), paroxysmal nocturnal hemoglobinuria
   - Cancer
   - Nephrotic syndrome
   - Atrial fibrillation
   - Myocardial infarction
   - Heparin-associated thrombocytopenia
   - Thrombotic thrombocytopenic purpura (TTP)
4. Increased hepatic synthesis of coagulation factors and reduced anticoagulant synthesis
   - Oral contraceptive pill
   - Hyperestrogenic states (pregnancy and postpartum)
5. Release of procoagulant substances: Disseminated cancers
6. Tissue injury: Surgery, fracture, extensive burns
7. Reduced endothelial PGI_2: Old age
8. Endothelial injury: Homocysteinemia
9. Fibrinolytic system defects
10. Unknown: Smoking, obesity, sickle cell anemia

INHERITED HYPERCOAGULABLE STATES

Individuals with inherited hypercoagulable states are at a risk of thromboembolism. But not all patients develop thrombotic episodes unless some acquired factors precipitate it. They usually develop due to genetic mutations which affect one of the naturally occurring anticoagulant mechanisms.

Clinical presentation: It may present with one or more of the following features.

- Thrombosis usually develops at young age (less than 45 years)
- Recurrent thromboembolism
- A family history of thromboembolic episodes is usually present
- Almost always thrombosis develops in the venous system and at unusual anatomical sites like visceral veins.

The inherited (primary) hypercoagulable states are due to mutations that lead to either (1) **deficiency of a physiologic antithrombotic factor** or (2) **increased level of a prothrombotic factor** (Table 37.1).

Deficiency of Antithrombotic Factors

Antithrombin (AT) III Deficiency

- Antithrombin III is a physiological inhibitor of thrombin in circulation (see Fig. 33.7) and its **deficiency** results in **thrombus formation.**
- It is an autosomal dominant disorder, which has incomplete penetrance.
- Deficiency of antithrombin can be either quantitative or qualitative.
- The risk of a thrombotic event (usually venous) ranges from 20 to 80%.

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Protein C Deficiency

- **Proteins C and S** act as a complex (see Fig. 33.4), which **degrades factors Va and VIIIa.** When they are deficient, activated factor V and VIII are not neutralized and resulting activation of the clotting system, which promotes thrombosis.
- It may be homozygous or heterozygous type.
  - Homozygous protein C deficiency is a serious life-threatening condition. They present during neonatal period with thrombosis, purpura fulminans characterized by superficial thrombosis and necrotic skin lesions.
  - Heterozygous protein C deficiency may be asymptomatic or present with recurrent venous thrombosis at a young age.

Protein S Deficiency

Its presentation is similar to protein C deficiency.

Increased Prothrombotic Factors

Activated Protein C (APC) Resistance (Factor V Leiden)

- Activated protein C (APC) resistance is the most common genetic disorder associated with familial thrombophilia.
- Normally, protein C is activated by thrombin and gets converted into activated Protein C (APC). **APC** combines with protein S (PS) to form a complex which inhibits activated factor V and VIII (see Fig. 33.4).
- A **point mutation** (results in a glutamine to arginine substitution at position 506) in the **factor V gene** (this variant is known as factor V Leiden/*Leiden mutation* – it was first discovered in *Leiden* city of Netherlands) leads to synthesis of a **factor V variant** which has normal procoagulant activity but is resistant to **inhibition by activated protein** C (APC).
- It may be heterozygous or homozygous. In heterozygotes, the risk of thrombosis is 5-10 times greater, and in homozygotes it is 40-50 times compared to normal population.

Other Causes

- Excessively high levels of prothrombin may develop due to a **point mutation** in the **prothrombin gene** and is associated with threefold increased risk of thrombosis.
- Unusually high levels of fibrinogen, factor VII and factor VIII are associated with thrombosis.
- Some dysfibrinogenemia may result in abnormal fibrinogen associated with thrombosis.

ACQUIRED HYPERCOAGULABLE STATES

Acquired (secondary) hypercoagulable states constitute wide variety of diseases which may act as a precipitating factor in patients with inherited hypercoagulable states. The causes (Table 37.1 and Fig. 37.1) and mechanisms of acquired hypercoagulable states are quite variable.

Among the acquired hypercoagulability states, important and most common is antiphospholipid syndrome.

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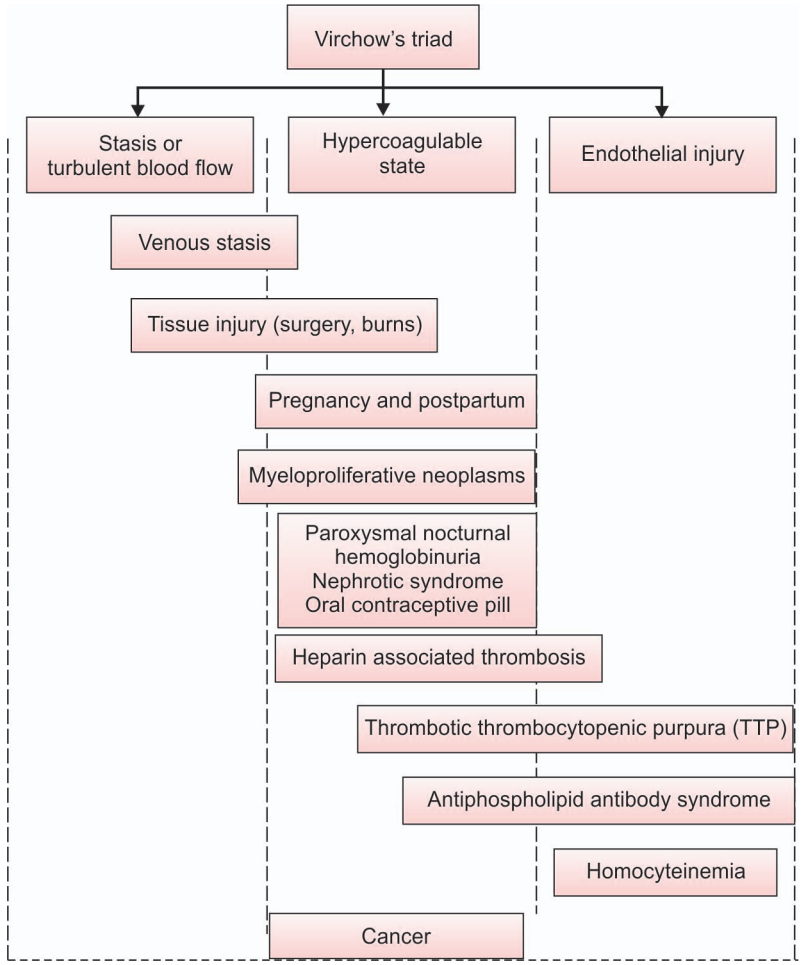


Fig. 37.1: Predominant mechanisms of thrombosis in few secondary hypercoagulable states based on Virchow's triad of thrombogenesis

Antiphospholipid Antibody Syndrome (APLA/APS)

- Antiphospholipid antibody syndrome is characterized by the presence of a family of antibodies in the patient's plasma known as antiphospholipid antibodies.
- The term **antiphospholipid antibody** is misleading because these antibodies react with plasma proteins which themselves bind and form complexes with negatively charged phospholipids (Fig. 37.2). So, in contrast to the terminology antiphospholipid there is no direct binding of antibodies to phospholipids.
- Many such plasma proteins (e.g. prothrombin, β_2-glycoprotein) bind to anionic phospholipids resulting in different antiphospholipid antibodies. Two important antibodies are **lupus anticoagulant antibody and anti-β_2 glycoprotein antibody.**

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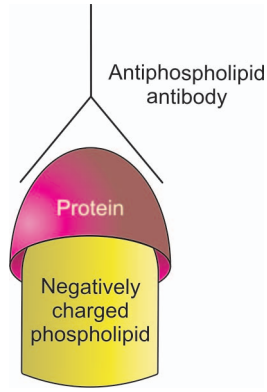


Fig. 37.2: Antiphospholipid antibody against plasma proteins bound to phospholipids

- **Lupus anticoagulant antibody:** It is named lupus as it was first detected in patients with systemic lupus erythematosus (SLE). It was called anticoagulant because it paradoxically **prolongs the phospholipid-dependent coagulation tests** *in vitro* (e.g. prolongation of APTT). However, the term lupus anticoagulant is a misnomer, because it is more frequently found in patients without lupus and is not anticoagulant and is associated with a hypercoagulable state rather than bleeding.
- **Antibodies against the phospholipid–β_2-glycoprotein complex:** It is one of the protein-anion phospholipid complexes which also bind to cardiolipin antigen. Since cardiolipin antigen is used in the serological test for syphilis, patients having this antibody may give a **false-positive serological reaction for syphilis.**

Types

Antiphospholipid antibody syndrome may be of **primary or secondary type**

- **Primary antiphospholipid syndrome:** They do not have any predisposing cause and their only manifestation is hypercoagulable state.
- **Secondary antiphospholipid syndrome:** These are found in association with autoimmune diseases, like systemic lupus erythematosus. Because of this association they were formerly known as lupus anticoagulant syndrome.

Clinical Features

- **Hypercoagulable state:** It is the commonest acquired hematologic cause of recurrent thromboembolic events. Depending on the site of thrombus it may present as pulmonary embolism (due to venous thrombosis of lower limb), pulmonary hypertension (due to recurrent subclinical pulmonary emboli), stroke or bowel infarction. Renal involvement may cause hypertension, renal microangiopathy and renal failure due to multiple glomerular capillary and arterial thromboses.

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- **Repeated spontaneous abortions:** Recurrent fetal loss is due to antibody-mediated inhibition of t-PA activity. Tissue plasminogen activator (t-PA) is necessary for the invasion of uterine blood vessels by placental trophoblastic tissue.
- **Thrombocytopenia**

Laboratory Tests

Coagulation tests

- APTT: Prolonged
- Factor VIII levels: Normal
- Prothrombin time: Normal
- Thrombin time: Normal
- Fibrinogen level: Normal

Dilute Russell's viper venom test (DRVVT)

Russell's viper venom (RVV) activates factor X leading to fibrin clot. Lupus anticoagulant prolongs clotting time by binding to RVV and preventing the action of RVV.

Confirmatory test

Antiphospholipid antibodies are detected by ELISA and radio immune assay (RIA).

Homocysteinemia

- An increased plasma concentration of homocysteine is known as homocysteinemia, which predisposes to atherosclerosis and thrombosis.
- The plasma homocysteine level may be genetically determined, but is also partly controlled by the dietary content of vitamin B_{12}, folate and pyridoxine.
- Deficiency of these dietary vitamins causes moderate degree of homocysteinemia.
- Mechanism of thrombosis is not clearly known. It may be due to:
  - Endothelial damage induced by homocysteine.
  - Linkage between homocysteine metabolites and various proteins, including fibrinogen.

SUMMARY

- Hypercoagulability state (thrombophilia) is a group of inherited or acquired conditions that are associated with increased tendency to develop thrombosis.
- Inherited causes include: activated protein C (APC) resistance (factor V Leiden), antithrombin III deficiency, protein C and S deficiency and dysfibrinogenemia.
- Acquired causes constitute a wide variety of diseases which may act as a precipitating factor in patients with inherited hypercoagulable state.
- One of the important and common acquired causes is antiphospholipid syndrome. It is characterized by the presence of different antibodies like lupus anticoagulant antibody and anticardiolipin antibody.

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SELF-ASSESSMENT EXERCISES

I. Short Note

Antiphospholipid antibody syndrome

II. Multiple Choice Questions

1. **Hypercoagulability due to defective factor V gene is called**
   A. Thrombotic thrombocytopenic purpura
   B. Leiden mutation
   C. Antiphospholipid syndrome
   D. Inducible thrombocytopenia syndrome
2. **The following is NOT associated with hypercoagulable state**
   A. Protein C resistance
   B. Antiphospholipid antibody
   C. Perforated gastric ulcer
   D. Nephrotic syndrome
3. **Most common genetic disorder associated with hypercoagulable state is**
   A. Protein C deficiency
   B. Protein S deficiency
   C. Factor V Leiden mutation
   D. Prothrombin gene mutation
4. **Antiphospholipid syndrome shows following features EXCEPT**
   A. Repeated spontaneous abortions
   B. Hypercoagulable state
   C. Thrombocytopenia
   D. Hypofibrinogenemia

ANSWERS

1. B **2.** C **3.** C **4.** D

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SECTION 4

Clinical Pathology

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CHAPTER 38 Anticoagulants and Collection of Blood

CHAPTER OUTLINE

- Anticoagulants
- Steps in Hematological Investigation

ANTICOAGULANTS

Blood coagulates when withdrawn from the vessel and anticoagulants are used to prevent blood from clotting. Commonly used anticoagulants are shown in Table 38.1.

Table 38.1: Commonly used anticoagulants

I.	**Calcium chelating agents**
	a. EDTA
	b. Double oxalate
	c . Sodium citrate solution
	d. Sodium fluoride
II.	**Heparin**

Calcium Chelating Agents

Most of the anticoagulants used in the laboratory act by binding calcium as an insoluble salt or soluble but un-ionized salt. Since calcium is essential for many of the steps in the clotting mechanism, chelation of calcium prevents clotting.

a. EDTA (Ethylene Diamine Tetra Aceticacid): Commonly disodium or tripotassium salts are used in the laboratory.

Action: They bind and remove free calcium ions from blood by chelation, thereby preventing coagulation.

Advantages: EDTA **preserves the morphology** of the blood cells even upto 3 hours and cell counts upto 12 hours after collection of the blood. Therefore, it is the **anticoagulant of choice** for all cell counts and peripheral smear. Tripotassium salts of EDTA are more soluble and are preferred for cell counters than that of less soluble sodium salt. EDTA is used in solid form at concentration of 2 mg/mL of blood.

Disadvantages: It is more expensive than oxalate, but this is usually outweighed by the advantages. It is **not suitable for coagulation studies.**

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Alterations induced by EDTA:

- **RBC:** Red cells may assume spherocytic or macrocytic form.
- **WBC:** Morphologic changes take place in the neutrophils within 30 minutes of blood collection.
- **Platelets:** They swell and disintegrate and a false decrease in their count may be observed.

Uses

- Cell counts: RBC count, total leukocyte count, eosinophil count, platelet count.
- Hemoglobin, HbF estimation and Hb electrophoresis.

b. Oxalate

- **Potassium oxalate:** Most often used for chemical analysis. Shrinks RBCs and therefore not recommended for packed cell volume (PCV), erythrocyte sedimentation rate (ESR), or peripheral smear.
- **Ammonium oxalate:** It causes swelling of RBCs and therefore not used for PCV, ESR or peripheral smear.
- **Double oxalate:** To balance the swelling effect of ammonium oxalate and the shrinking effect of potassium oxalate, the two are combined in a mixture in the ratio of 3 parts of ammonium oxalate to 2 parts of potassium oxalate. Morphology of the blood cells is not well preserved; hence it has now been replaced by EDTA. As it is toxic, it is not used as an anticoagulant for blood transfusion. It is used in concentration of 2 mg/mL of blood.

c. Sodium Citrate

Action: Sodium citrate binds with calcium ions in the blood to form a soluble calcium citrate complex thus preventing coagulation of blood.
Disadvantages: Since it is used as a liquid which results in dilution of cellular elements, it is not suitable for cell counts, Hb, etc.

Uses

- **Coagulation studies:** Either 3.8% trisodium citrate pentahydrate or 3.2% trisodium citrate dihydrate solution is used for coagulation studies. One part of anticoagulant is mixed with 9 parts of blood (1:9, i.e. ratio of 0.5 mL: 4.5 mL.) and thus, there is less dilution of the plasma.
- **ESR by Westergren method**: In the Westergren method of ESR estimation, 1 part of 3.8 percent sodium citrate is mixed with 4 parts of blood (e.g. 0.4 mL citrate and 1.6 mL blood/ 0.5 mL: mL of blood), thus maintaining a **ratio of 1:4 between citrate and blood**.
- **Blood Bank:** ACD Solution (acid citrate dextrose solution) was used as an anticoagulant in blood banking. This has been replaced by citrate phosphate dextrose adenine (CPDA).

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d. Sodium Fluoride

Sodium fluoride acts an antiglycolytic agent which inhibits the use of glucose by blood cells.

Use: It is used for estimation of **blood sugar.**

Heparin

Heparin acts as an anticoagulant by preventing conversion of prothrombin to thrombin. It is used when almost instantaneous anticoagulation is required and to prevent any chances of hemolysis after the blood is drawn. Syringe is heparinised by taking 1 drop of heparin to wet the inner surface of the syringe and the blood is collected.

Disadvantages

- Expensive
- Causes clumping of platelets and leukocytes, so it is not used for automated cell counters.
- Imparts bluish discoloration to the background of peripheral blood smears.
- Prevents coagulation for only a limited period of time and its effect slowly disappears as the heparin is metabolized or neutralized in the blood.

Uses

- Osmotic fragility test done for diagnosis of spherocytosis.
- Red cell enzyme studies like G6PD and PK deficiency.
- Electrolyte estimation.
- Arterial blood gas analysis.

STEPS IN HEMATOLOGICAL INVESTIGATION

- For an accurate testing and result of any laboratory investigation, it is essential to prepare the patient for the concerned test, collect the specimen and handle it properly.
- After the samples are collected, they should be labeled properly with name, age, sex, etc. of the patient.
- The sample should be sent to the laboratory as early as possible with the properly filled requisition form (along with the investigation required).
- Most of the tests should be performed within 1-2 hours after the collection of the blood.

Collection of Blood

The blood for the tests is collected in vacuum tubes which have a colored top (Table 38.2 and Fig. 38.1). It is very essential to use appropriate vacuum tubes with proper ratio of anticoagulant and the blood for specific tests. For example, the ratio of anticoagulant to blood is 1: 9 (sodium citrate-anticoagulant) for coagulation studies.

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Table 38.2: Types of vacuum tubes, its color, content and uses

Color of the top	*Anticoagulant*	*Uses*
Red	No chemicals-plain	Serum for various studies
Purple or lavender	EDTA	Routine hematological tests
Light blue	Sodium citrate	Coagulation studies
Gray	Fluoride	For glucose estimation
Green	Heparin	Osmotic fragility test, red cell enzyme study
Black	Citrate	Erythrocyte sedimentation rate



Fig. 38.1: Different types of vacuum tubes

Type of Blood Sample

- Certain blood chemistry tests require fasting blood sample. The term fasting means, overnight fasting (8 to 10 hours) so that the patient is in the basal metabolic state. Fasting minimizes the effects of ingested substances on the results of various laboratory tests (like lipid profile). Intake of water is allowed during fasting.
- The blood sample required may be whole blood, serum or plasma.

1. Whole Blood

Uses

- For complete hemogram (hemoglobin, ESR, platelet count, reticulocyte count, and peripheral smear evaluation).
- Osmotic fragility test, estimation of HbF, Hb electrophoresis, Coombs test, etc.
- For biochemical investigations like blood glucose.

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2. Serum

Serum is obtained by allowing the blood to clot in a tube or in a vial (without adding any anticoagulant). It requires about 1 hour for the retraction of clot to release serum. When the clot retracts, straw colored serum admixed with few RBCs is obtained. Pour the serum into another tube and centrifuge at 3000 RPM for 15 minutes. RBCs will settle down and clear serum is obtained. Serum is devoid of fibrinogen and major fraction of clotting factors (like XI, VIII, IX, X, V, VII and II).

Uses

- Estimation of bilirubin, creatinine, uric acid, proteins, albumin, globulin and A/G ratio, etc.
- Serum enzyme levels like alkaline phosphatase, acid phosphatase, aspartate (AST/SGOT) and alanine aminotransferase (ALT/SGPT).
- Serum electrophoresis of proteins, lipoproteins and immunoglobulins.

3. Plasma

Plasma is obtained by centrifugation of the anticoagulated blood (e.g. citrated blood collected for coagulation studies). Red blood cells form the sediment and the supernatant is the plasma.

Uses

- Coagulation studies: Prothrombin time (PT), activated partial thromboplastin time (APTT), thrombin time.
- Assay of various coagulation factors like factor VIII, IX.
- Assay of FDP and D-dimer as in disseminated intravascular coagulation.
- Confirmation of hemoglobinemia where plasma is colored red (e.g. PNH).

Methods of Blood Collection

Blood may be collected as follows:

Venous blood

- It is usually collected from antecubital vein either by a disposable syringe or vacuum tubes.
- Clean the skin of the antecubital area of the arm with an alcohol swab and allow it to dry.
- Apply a tourniquet 2 to 4 inches above the selected venous puncture site for no longer than 1 minute and ask the patient to close the fist. The tourniquet provides a barrier against venous blood flow and makes the vein prominent.

By syringe

- Insert the needle of the syringe into the vein. Appearance of blood in the hub of the needle confirms that the needle is in the vein.

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- Ask the patient to open the fist as blood enters into the syringe and release the tourniquet. When the required amount of blood is collected in the syringe, withdraw the syringe with the needle from the vein. Place the spirit swab at the skin puncture site and apply mild pressure.
- Discharge the blood from the syringe into appropriate vial.
- Pour one drop each on 2 to 3 glass slides and prepare peripheral blood smears.

By vacuum tubes

- The proper vacuum tube is selected and placed in a reusable plastic holder. A disposable needle is attached by screwing the threaded needle hub to the holder. The vacuum tube is then inserted into the holder until the top of its stopper is level with the marked guideline which causes the tube to recede.
- Patient's arm is placed slightly in a vertical position. Note that for vacuum tubes, venipuncture should not be performed in a horizontal plane because it may result in the backflow of blood.
- The needle is inserted into the vein similar to syringe technique and the base of the collection tube is pushed forward breaking the vacuum seal. Time is allowed for the vacuum tube to fill.
- Ask the patient to open the fist. Release the tourniquet and withdraw the needle along with the vacuum tube.

Capillary blood

Capillary blood may be collected either from the finger or heel.

Finger prick method

- Clean the palmar aspect of tip of the ring finger with alcohol swab and allow it to dry.
- Using sterile disposable lancet/needle, prick on the lateral side of the tip of the finger so that blood starts flowing. Wipe the first two drops.
- The free flowing blood is collected in the hemoglobinometer pipette for hemoglobin estimation, total WBC count, platelet count, etc.
- Peripheral smears are also prepared by placing a drop of blood on one end of the glass slide.

Heel prick method

This method is usually employed for collecting blood from infants.

- Gently warm the heel by gentle rubbing. Clean the lateral or medial aspect of the plantar surface of the heel using an alcohol swab.
- Prick by using a sterile disposable lancet/needle.
- Collect blood for hemoglobin estimation, total WBC count and prepare the peripheral smears.

Arterial blood

Arterial blood is usually required for blood gas analysis and is collected from the femoral artery.

- First feel the pulsations of the femoral artery below the inguinal ligament.
- Clean the area and collect blood with a disposable needle and syringe.

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Complications Encountered During Blood Collection

- Ecchymosis and hematoma at the site of puncture.
- Syncope or fainting is one of the frequent complications. If patient begins to develop syncope, immediately remove the needle and make the patient lie down in a head low position.
- Failure to get blood because the needle might not have entered the vessel. In obese individuals, it is often difficult to locate the vein.
- Hemolysis of blood sample due to narrow bore of needle, quick withdrawal of blood with excessive suction or contamination of syringe by water. Hemolyzed blood sample is not suitable for testing.

Labeling

Immediately after collection (either in the laboratory or ward), the specimen should be labeled. The minimal information that must be provided on each tube is: patient's full name, age, sex, unique identification number, date and time of collection.

Laboratory Requisition Form

The laboratory requisition form should be filled properly. It is essential that it should contain in brief signs and symptoms of the patient.

SUMMARY

- Anticoagulants are used to prevent coagulation of blood collected for various laboratory investigations.
- Commonly used anticoagulants are either calcium chelating agents (EDTA, sodium citrate solution, double oxalate, and sodium fluoride) or heparin.
- The blood sample required may be whole blood, serum or plasma.
- Serum is obtained by allowing the blood to clot in a tube/vial without an anticoagulant and plasma by centrifugation of the anticoagulated blood.
- The blood is collected in appropriate vacuum tubes which have a colored top.
- Immediately after collection of blood, sample should be labeled and sent to the laboratory with adequately filled requisition form.

SELF-ASSESSMENT EXERCISES

I. Short Notes

1. Anticoagulants
2. Discuss anticoagulants used in hematology
3. Double oxalate mixture and its uses
4. Differences between plasma and serum
5. Anticoagulants for ESR estimation
6. Complications of venipuncture

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II. Multiple Choice Questions

1. **The anticoagulant of choice for coagulation studies is**
   A. EDTA
   B. Heparin
   C. Trisodium citrate
   D. Double oxalate
2. **The anticoagulant of choice for blood cell counts is**
   A. EDTA
   B. Sodium fluoride
   C. Sodium citrate
   D. Double oxalate
3. **The anticoagulant used for blood sugar estimation is**
   A. EDTA
   B. Sodium fluoride
   C. Sodium citrate
   D. Double oxalate
4. **Anticoagulant suitable for storing blood for transfusion is**
   A. Dextrose, heparin and citrate
   B. Dextrose, phosphate and citrate
   C. Dextrose, EDTA and saline
   D. Glucose, phosphate and oxalate

ANSWERS

1. C **2.** A **3.** B **4.** B

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CHAPTER 39

Hematopoiesis

CHAPTER OUTLINE

- Introduction
- Erythropoiesis
- Myelopoiesis
- Megakaryopoiesis

INTRODUCTION

Formation of red cells, white cells and platelets occurs in the bone marrow. Their progenitors and precursors proliferate, mature and the formed elements are then released into the circulation. The precursors and various immature cells (erythropoiesis, myelopoiesis, megakaryopoiesis and lymphopoiesis) have characteristic morphologic appearances. The various stages of development of red cells, white cells and platelets and their morphological features are important for the evaluation of bone marrow.

ERYTHROPOIESIS

Erythropoiesis is the process by which marrow hematopoietic stem cells pass through several stages, proliferate, differentiate and mature to form red blood cells. Progenitors and red cells are together known as *erythron*. As the maturation proceeds, the features observed are shown in Table 39.1.

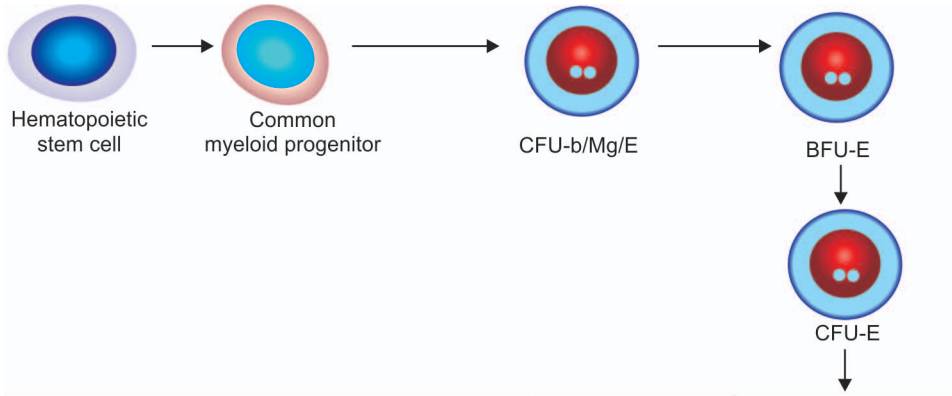
Table 39.1: Changes observed in erythroid cells as they mature

Cellular component	*Characteristics*
Size	Progressive reduction
Nucleus	First condenses, then pyknotic (in late normoblast) and finally extruded from the cell during reticulocyte stage
Hemoglobin synthesis	Occurs in the cytoplasm
Cytoplasmic staining	
Before the appearance of hemoglobin	Basophilic
When hemoglobin appears	Polychromatic and stained by both acidic and basic dyes
When hemoglobin synthesis is complete (late normoblast)	Acidophilic
Functional maturation	When matures capable of carrying oxygen

Stages of erythropoiesis: The normal lifespan of red cells is 110 to120 days and about 1% of the red cells are replaced daily. In the bone marrow, erythroid precursors form islands around reticulum cells which supply iron to developing normoblasts. The various stages of erythropoiesis are shown in Fig. 39.1.

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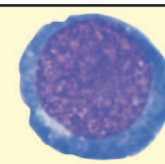
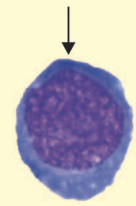
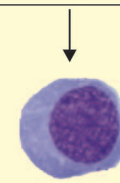
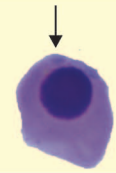
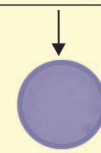
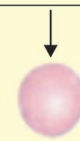
Morphological features		
Shape: Round Size: 10-16 μm Cytoplasm: Dark basophilic cytoplasm Nucleus: Central nucleus with slightly clumped chromatin, 2-5 nucleoli.	Pronormoblast	
Shape: Round Size: 8-18 μm Cytoplasm: Less basophilic than pronormoblast Nucleus: Coarse chromatin, nucleoli not seen.	Basophilic (early) normoblast	
Shape: Round Size: 8-12 μm Cytoplasm: Polychromatic due to hemoglobinization of cytoplasm Nucleus: Deep staining and coarse chromatin, no nucleoli	Polychromatic (intermediate) normoblast	
Shape: Round Size: 8-10 μm Cytoplasm: Well hemoglobinized cytoplasm Nucleus: Begins to condense and becomes pyknotic, eccentric or partially extruded.	Orthochromatic (late) normoblast	
Cells stain bluish and are larger than normal red cells Demonstrated by supravital staining (brilliant cresyl blue or new methylene blue)	Reticulocyte	
Normal RBC is biconcave disc 7-7.7 μm in diameter	Red blood cell	

Fig. 39.1: Stages of erythropoiesis

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Regulation of erythropoiesis: To a large extent, erythropoiesis is dependent upon

- Various nutrients like iron, proteins and vitamins
- Erythropoietin

Erythropoiesis is regulated by erythropoietin (EPO) synthesized in the kidney. EPO production is increased in response to hypoxia/reduced oxygen tension (e.g. due to anemia, lung disease). *Erythropoietin* acts on EPO receptors present on erythroid precursors (see Fig. 18.1). Failure of erythropoietin production is a major causative factor for anemia of chronic renal failure. Excessive EPO results in polycythemia secondary to renal tumors.

Dyserythropoiesis is defective erythropoiesis in which abnormal red cells are produced. These abnormal cells are either destroyed in the bone marrow or enter circulation and undergo hemolysis. The dyserythropoiesis is found in conditions like megaloblastic and congenital dyserythropoietic anemias. Morphologically, the normoblasts show nuclear abnormalities such as:

- Nuclear budding
- Nuclear bridges
- Abnormal mitosis
- Premature nuclear extrusion.

The red cells show basophilic stippling and Howell-Jolly bodies (see Fig. 42.5) in the cytoplasm.

Ineffective erythropoiesis: The term ineffective erythropoiesis is used when developing erythroid precursors die within the marrow without producing mature cells.

Assessment of erythropoiesis: Total amount of effective erythropoiesis can be assessed by examining bone marrow, hemoglobin and reticulocyte count. The reticulocyte count is increased in proportion to the degree of anemia when eythropoiesis is effective and is decreased when erythropoiesis is ineffective.

Reaction of Marrow

- **Normoblastic** is the term used for normal maturation of red cell precursors.
- **Megaloblastic maturation** is seen with vitamin B_{12} and folic acid deficiency (Chapter 4).
- **Micronormoblastic maturation** is observed in iron deficiency anemia (Chapter 3). This is characterized by small normoblasts with persistent basophilia of cytoplasm and fraying of cytoplasmic borders.

MYELOPOIESIS

Myelopoiesis (granulopoiesis) results in production of neutrophils, eosinophils, basophils and monocytes. During myelopoiesis, as the maturation proceeds the changes observed are shown in Table 39.2.

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Table 39.2: Changes observed in granulopoietic cells as they mature

Cellular component	*Characteristic features*
Size	Cells and nuclei from myeloblast onwards become smaller
Cytoplasmic granules	Azurophil granules appear in promyelocytes which progressively diminish until metamyelocyte stage and then granules remain constant. Specific granules are observed from myelocyte onwards
Mitosis	Occurs in blast, promyelocyte and myelocyte stage. From metamyelocyte stage onwards only maturation takes place
Nucleus	Chromatin progressively becomes coarser and deeply stained and nucleus becomes segmented

Stages of myelopoiesis: The various stages in myelopoiesis are shown in Fig. 39.2.

Granulocyte pools: Mature granulocytes are distributed in two pools.

- Circulating granulocyte pool
- Marginal granulocyte pool where cells are present along the margin of the capillaries.

Increased physiological demand for granulocytes results in migration of cells from marginal pool into the circulation and to the site of infection.

Regulation of myelopoiesis: Myelopoiesis is regulated by various growth factors and cytokines, most important being granulocyte-macrophage colony-stimulating factor (GM-CSF) and granulocyte colony-stimulating factor (G-CSF).

Development of Eosinophils and Basophils

Different stages of myelopoiesis for eosinophils and basophils are similar to that of neutrophils except that the myelocytes and metamyelocytes demonstrate eosinophilic and basophilic granules respectively.

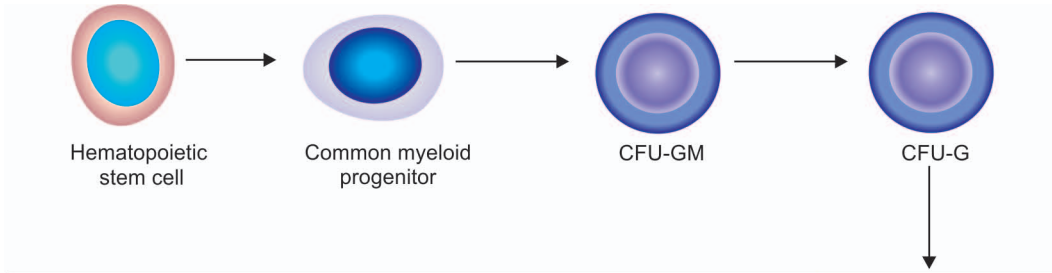
MEGAKARYOPOIESIS

Megakaryopoiesis (thrombopoiesis) is the process of platelet formation. The various stages of megakaryopoiesis are shown in Fig. 39.3.

- The megakaryocyte is an easily identifiable cell in megakaryopoiesis because of its large size. They undergo nuclear division without cytoplasmic division (endomitosis). In the bone marrow megakaryocytes are situated close to the marrow sinusoids.
- Platelets are the fragments of the cytoplasm of megakaryocytes and are formed by pinching off their cytoplasm. They are released in groups (called proplatelets) into the sinusoids and reach circulation. Each megakaryocyte releases 1000 to 4000 anucleate platelets. In a Romanowsky (Leishman, Giemsa) stained smear, platelets appear as pale blue colored structures of varying sizes having purple granules.
- Normal platelet count: 150-450 × 10^9/L
- Normal lifespan: About 4-10 days

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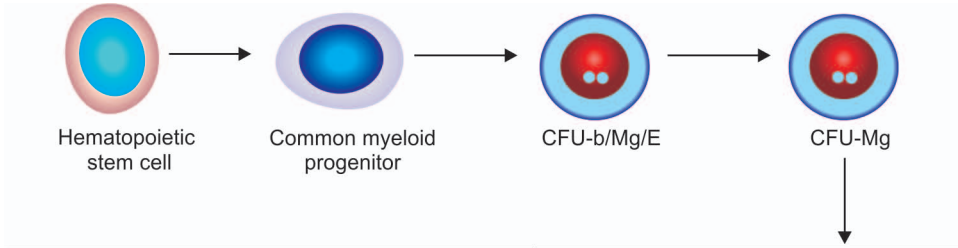


Size: 15-25 μm Cytoplasm: Basophilic, usually non-granular Nucleus: Central, round, reticular chromatin network. Nucleoli: 3-5	Myeloblast	
Size: 20-30 μm Cytoplasm: Purplish-red azurophilic granules Nucleus: Central/eccentric, round with fine chromatin pattern. Nucleoli: 1-2	Promyelocyte	
Size: 12-15 μm Cytoplasm: Fine specific granules Nucleus: Flattened on one side with chromatin coarser than in promyelocyte. Nucleoli: Absent	Myelocyte	
Size: 10-15 μm Cytoplasm: Numerous fine granules (specific and primary) Nucleus: Eccentric kidney shaped nucleus, coarser chromatin than that of myelocyte.	Metamyelocyte	
Size: 10-14 μm Cytoplasm: Granular (specific and primary) Nucleus: U-shaped or deeply indented.	Band form	
Size: 10-12 μm Cytoplasm: Abundant fine granules (specific and primary) and alkaline phosphatase. Nucleus: 2-5 lobes of the nucleus joined by chromatin thread.	Polymorphonuclear leukocyte	

Fig. 39.2: Stages of myelopoiesis

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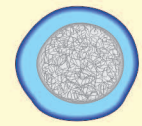
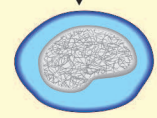
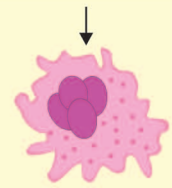
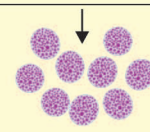
Size: 15-20 μm Cytoplasm: Basophilic cytoplasm Nucleus: Round Nucleoli: Single to multiple nucleoli	Megakaryoblast	
Size: 20-30 μm Cytoplasm: Abundant, less basophilic cytoplasm Nucleus: Oval and elongated	Promegakaryocyte	
Size: 35-60 μm, largest cell in the bone marrow Cytoplasm: Borders irregular and budding of platelets from the cytoplasmic border. Rich in granules. Nucleus: Compact and dense, multi-lobated and pushed to one side.	Megakaryocyte	
Size: 2-3 μm, Anucleated pale blue colored structures with purple granules	Platelets	

Fig. 39.3: Stages of megakaryopoiesis

Regulation of megakaryopoiesis: Thrombopoiesis is regulated by cytokines and growth factors, most important being thrombopoietin (TPO), which is synthesized in liver. Other cytokines include IL-3, IL-6 and IL-11.

Functions of platelets: They play an important role in:
- Hemostasis
- Coagulation
- Thrombosis.

SUMMARY

- Erythropoiesis is the process by which marrow hematopoietic stem cells pass through several stages to form red blood cells.
- The various morphologically recognizable stages in erythropoiesis are pronormoblast, basophilic normoblast, polychromatic normoblast, orthochromatic normoblast, reticulocyte and mature red cell.

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- The maturation process in erythropoiesis is characterized by the decrease in size of cell, condensation and complete extrusion of nucleus and gradual hemoglobinization of the cytoplasm.
- The various stages in myelopoiesis are myeloblast, promyelocyte, myelocyte, metamyelocyte, band form and mature granulocyte. The maturation process in myelopoiesis is characterized by appearance of specific and primary granules.
- In megakaryopoiesis, the stages include megakaryoblast, promegakaryocyte, megakaryocyte and platelets.
- Platelets are the fragments of the cytoplasm of megakaryocytes and appear in the peripheral smear as pale blue colored structures with purple granules.

SELF-ASSESSMENT EXERCISES

I. Short Notes

1. Different stages of normoblastic erythropoiesis
2. Extramedullary hematopoiesis
3. Different stages of normal myelopoiesis
4. Different stages of megakaryopoiesis

II. Multiple Choice Questions

1. **The precursor of platelets is**
   A. Endothelial cell
   B. Normoblast
   C. Megaloblast
   D. Megakaryocyte
2. **Erythropoietin is synthesized in**
   A. Liver
   B. Kidney
   C. Spleen
   D. Bone marrow
3. **Normal platelet count is**
   A. 50-150 × 10^9/L
   B. 150-450 × 10^9/L
   C. 450-600 × 10^9/L
   D. 600-750 × 10^9/L

ANSWERS

1. D **2.** B **3.** B

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CHAPTER 40 Hemoglobin Estimation

CHAPTER OUTLINE

- Methods of Hemoglobin Estimation
- Colorimetric Methods
- Other Methods

METHODS OF HEMOGLOBIN ESTIMATION

One of the important features of anemia is reduction in the red cell mass. Red cell mass can be estimated by measuring hemoglobin (Hb).

There are several methods for Hb estimation utilizing different principles (Table 40.1). The commonly used methods include colorimetric method namely Sahli's and cyanmethemoglobin method.

Table 40.1: Methods of hemoglobin estimation

Method	*Principle*
A. Colorimetric method	These methods are based on measuring the **color** of hemoglobin or a hemoglobin derivative in the blood either visually or by photocolorimetry. The optical density of a colored solution is directly proportional to the concentration of the colored material in the solution.
1. Visual colorimetric method	
• Sahli's method or acid hematin method	
• Alkaline hematin method	
2. Photocolorimetric (photoelectric) method	
• Cyanmethemoglobin (HiCN) method	
• Automated analyser	
• Hemo-cue blood hemoglobin system	
• Oxyhemoglobin method	
• Haldane method	
B. Physical method	Specific gravity
C. Chemical method	Iron content of hemoglobin
D. Gasometric method	Oxygen combining capacity of hemoglobin

COLORIMETRIC METHODS

Visual Colorimetric Method

Sahli's Method or Acid Hematin Method

This is the most popular colorimetric method.

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Principle

When blood is added to N /10 HCl, **hemoglobin is converted into acid hematin.** The resulting brown color of acid hematin is matched against the standard brown color of the comparator. The intensity of the brown color depends on the amount of acid hematin produced, which in turn depends on the amount of hemoglobin in the blood sample.

Apparatus (Fig. 40.1)

It consists of:

- Hemoglobin pipette which has one mark of 0.02 mL (20 cu mm).
- A comparator which has a flat surface and a standard brown color.
- A square/round tube with markings of hemoglobin percentage (100% = 14.5 gm/dL) on one side and hemoglobin (in gm) on the other side.
- Glass rod for mixing the blood in the square/round tube.
- A dropper to take and transfer N/10 HCl and distilled water into the square tube.

Note: Sahli's hemoglobinometer with square tube and flat comparator surface is preferable to round tube.



Fig. 40.1: Sahli's hemoglobinometer consisting of Hb pipette, comparator, round tube, glass rod and dropper

Technique

- Take N/10 HCl with a dropper into the hemoglobinometer tube up to the lowest mark.
- Draw blood up to 20 cu mm (0.02 mL) mark of Hb pipette and wipe off the excess blood at nozzle of the pipette with moist cotton. Blood may be taken either from a finger prick or an EDTA vial.

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- Blood in the Hb pipette is blown into the square tube of hemoglobinometer. Ensure that the entire blood inside the Hb pipette is transferred to the tube.
- Wait for 10 minutes for the acid to act on the RBCs which causes lysis of RBCs liberating hemoglobin. The acid converts Hb to acid hematin. (About 95% of Hb is converted at the end of 10 minutes, 98% of the color develops at the end of 20 minutes and the maximum color is reached after about 1 hour).
- With the help of a glass rod, mix the acid hematin.
- Match the color of the solution in the tube with that of the comparator by holding against the natural light or fluorescent tube light.
- Observe the following which interfere with matching and give wrong reading:
  - Remove the glass rod from the solution in the tube.
  - Keep the tube inside the comparator in such a way that the markings do not interfere with the matching exercise.
- If the solution in the tube is darker, add distilled water with a dropper and stir the solution with a glass rod. This process is continued till the color of the solution exactly matches the color of the comparator.
- Take the tube out and remove the glass rod. Take the reading from the lower meniscus.
- Hemoglobin is recorded as gram/dL (or gm%).

Sources of error

a. Technical errors: These are errors inherent in handling the sample.

- **Improper collection:**
  - Improper venipuncture technique may produce hemoconcentration resulting in high hemoglobin value.
  - In case of finger prick collection, improper technique or excessive squeezing results in tissue fluid contaminating capillary blood with corresponding error in the Hb value.
- **Improper mixing of blood sample:**
  - If blood is taken from EDTA vial it should be mixed properly before filling the pipette. If not, depending on whether the plasma or the sedimented RBCs have been aspirated in the Hb pipette, it may give rise to false low or high hemoglobin value.
- **Insufficient time for formation of hematin:** A minimum of 10 minutes is required for almost complete conversion of Hb to acid hematin. If reading is taken before, it can give false reading.
- **Time delay:** The brown color of acid hematin is not stable and the color gradually starts fading. Thus, undue delay in taking the reading results in errors.

b. Visual errors:

Comparison of colors is very subjective and can vary from person to person. Hence, the results may not be accurate.

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c. Errors inherent in the equipment

- The accuracy of different equipments is not uniform. If Hb pipette has not been accurately calibrated at 0.02 mL it can give rise to wrong values. Calibration of pipettes will reduce the errors.
- Color comparators can affect the reading. If the glass blocks of the comparators are old or faded, it may give an inaccurate value.

Disadvantages of the method

- Apart from the above technical errors, it is **not an accurate method.**
- Hydrochloric acid **does not convert all hemoglobins** (like carboxyhemoglobin, methemoglobin and sulphemoglobin) into acid hematin. Thus, Hb value obtained may be slightly lower than the actual Hb.
- **Non-hemoglobin substances** such as protein, lipid and cell stroma **interfere** with the color of blood diluted with acid and hence give false results.

Advantages of the method

- Simple and easy method
- Cheap.

Normal range

Depends on the instrument used.

Males	13-17 gm/ dL
Females	12-15 gm/dL
Newborns	14-19 gm/dL

Significance

- **Low Hb values:** Low hemoglobin values are found in **anemias.** Hemoglobin level is directly proportional to the severity of anemia. Morphological typing of the anemia can be done with the help of peripheral smear examination.
- **High Hb values:** High hemoglobin values (more than 18.5 gm/dL in males and 16.5 gm/dL in females) are seen in polycythemia (Chapter 18).

Alkaline Hematin Method

Principle: In this method the Hb is converted to alkali hematin by the addition of a strong alkali (N/10 NaOH). The alkali hematin formed is brown in color and is read against comparator standards or in a colorimeter. This method is not in use.

Note:

- Unlike Sahli's method, carboxyhemoglobin and sulphemoglobin are converted to alkali hematin.
- Fetal hemoglobin is resistant to denaturation by alkali and this method is used to determine the level of fetal hemoglobin in blood.

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Photoelectric (Photocolorimetric) Method

Cyanmethemoglobin (Hemiglobincyanide; HiCN) Method

Cyanmethemoglobin is the most accurate, convenient, readily available and preferred method for estimation of hemoglobin concentration. It is the standard method used in most of the centers.

Principle

Blood is diluted in a stable standard solution of potassium ferricyanide and potassium cyanide. **Hemoglobin is first oxidized to methemoglobin** (Hi) by the potassium ferricyanide. The cyanide ions (CN^-) of potassium cyanide convert **methemoglobin (Hi) to stable cyanmethemoglobin** (HiCN). The cyanmethemoglobin has a broad absorption, maximum being at a wavelength of 540 nm. Thus, the color of this solution is compared against a standard HiCN solution of known Hb value in a spectrophotometer or photoelectric colorimeter at 540 nm.

Apparatus

Photoelectric colorimeter with a green filter or spectrophotometer at a wavelength of 540 nm is used.

Reagents

The diluent is detergent-modified Drabkin solution, the composition of which is shown in Table 40.2.

Table 40.2: Constituents of Drabkin solution

Constituent	*Quantity*
Potassium ferricyanide	400 mg
Potassium cyanide (KCN)	100 mg
Potassium dihydrogen phosphate (anhydrous)	280 mg
Non-ionic detergent *(Sterox SE)*	1 mL
Distilled water	2 liters

Drabkin solution should be clear and pale yellow and when measured in the photoelectric colorimeter should give zero reading at 540 nm against water blank. The detergent enhances lysis of RBCs and decreases turbidity due to precipitation of proteins.

Technique

- Take 5 mL of Drabkin solution in a test tube.
- Take twenty microliter (0.02 mL) of blood in a hemoglobin pipette (similar to Sahli's method) and add it to the above test tube. Rinse the Hb pipette at least twice by drawing in Drabkin solution.
- Thoroughly mix the blood with Drabkin solution.
- Allow it to stand for 10 minutes at room temperature.

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- Switch on the photoelectric colorimeter at 540 nm or with an appropriate filter (green) colorimeter.
- Adjust optical density (OD) at 0 using Drabkin solution as blank.
- Pour test solution into the cuvette and note down OD of the test sample.
- OD of the standard cyanmethemoglobin solution is taken against the blank.

$$\textbf{Hb} = \frac{OD\ of\ test\ sample \times concentration\ of\ standard^{*} \times \mathbf{250}^{**}}{OD\ of\ standard \times \mathbf{1000}^{***}}$$

* Conc. of standard is given for each standard, e.g. 50 mg/100 mL.
** Since 0.02 mL of blood has been added to 5 mL Drabkin, dilution is 1 in 250.
*** Divide by 1000 to get the value in gm%.

The standard must be kept in a dark place while not in use and should be discarded at the end of the day.

Advantages of the method

- Hb value obtained is **accurate** since almost all forms of hemoglobin (hemoglobin, oxyhemoglobin, methemoglobin, carboxyhemoglobin, but not sulphemoglobin) are converted into cyanmethemoglobin.
- Being a colorimetric method there is direct comparison with HiCN Standard. Thus, **visual error** during matching the color like in Sahli's method is **eliminated.**
- Cyanmethemoglobin is a stable compound and **color does not change with time.** So readings can be made at the operator's convenience.
- **Easy** to perform the test.
- The **standard is stable** and certified cyanmethemoglobin standards are available.
- Reagents are readily available.

Disadvantages of the method

- **Turbidity interferes with the reading:** Any turbidity due to abnormal plasma proteins, hyperlipidemia, high WBC count or fat droplets or in the Drabkin solution itself alters value and therefore inaccurate Hb values are obtained.
- **Poisonous diluents:** Potassium cyanide in the solutions is poisonous, though it is present only in a very low concentration. Hence the reagents should be handled carefully.
- **Explosive reagent:** Explosion can occur if undiluted reagents are poured in the sink. Hydrogen cyanide is released by acidification and the gas, if it accumulates, can result in explosion. Reagents and samples should be disposed off along with the running water in the sink.

Precautions

- Do not pipette Drabkin solution by mouth. Use either suction bulb or auto dispenser.
- Hemoglobin standard should be used that is supplied by International Committee for standardization of hematology.

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Automated Analyser (Cell Counter)

These instruments measure not only hemoglobin but also various parameters like cell counts, PCV and absolute values (MCV, MCH and MCHC).

Hemoglobin estimation

- RBCs are lysed by the lysate and the color is matched against the inbuilt standard color and the Hb value is obtained.
- Most of the cell counters utilize a cyanide free bio-degradable reagent.
- These give an accurate result, provided these analysers are frequently standardized. However, the standards are expensive.

Hemo-cue Blood Hemoglobin System

It is a WHO approved method for Hb estimation in blood donors.

Principle

The reaction in the cuvette is a modified azidemethemoglobin reaction. The microcuvette contains three reagents (Table 40.3) in dried form which convert Hb into azidemethemoglobin (HiN3). The photometer uses a double wavelength measuring method, 570 nm and 880 nm (for compensation of turbidity) and determines the Hb.

Table 40.3: Constituents and action of reagents in microcuvette

Constituent	*Action*
Sodium deoxycholate	Hemolyses the RBC and releases the hemoglobin (Hb)
Sodium nitrite	Converts Hb (ferrous; Hb) to methemoglobin (ferric; Hi)
Sodium azide	Converts methemoglobin (Hi) to azidemethemoglobin (HiN3)

Apparatus (Fig. 40.2)

The hemo-cue blood hemoglobin system consists of:

- Disposable microcuvette with reagent in dry form
- Microcuvette holder
- Single purpose derived photometer.

Technique

- Apply microcuvette to a drop of blood. The exact amount of blood is drawn into the microcuvette by capillary action (capillary, venous or arterial blood can be used).
- Wipe off any excess blood from the sides of microcuvette.
- Place microcuvette in the cuvette holder and insert it into the photometer.
- The results are automatically displayed.

Advantages

- Quick and easy method
- It is a single use device. Sterile and safe for blood donors.
- It can be used for out-door blood donation camps.

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Fig. 40.2: Hemo-cue blood hemoglobin apparatus

Oxyhemoglobin Method

Principle: In this method, 0.04% liquid ammonia (ammonium hydroxide) is used to lyse the red cells. Oxyhemoglobin formed is then read in the colorimeter.

Haldane Method

Principle: In this method, red cells are lysed and carbon monoxide is added which converts hemoglobin to carboxy-hemoglobin. The color is compared with that of a given standard.

OTHER METHODS

Physical Method

Copper sulfate specific gravity method: It is an indirect reliable method used mainly for determination of hemoglobin in blood donors. In this method, standard copper sulfate solutions of specific gravity ranging from 1.052 to 1.055 are prepared. In blood banks, a solution of specific gravity of 1.053 is used for determining the hemoglobin level of 12.5 gm/dL for selecting the donor. One drop of blood is allowed to fall from a height of 1 cm above the surface of copper sulfate solution. If the drop of blood remains on the surface or rises from the bottom of the solution, then the hemoglobin is less than 12.5 gm/dL and if it sinks, it is equal to or higher than 12.5 gm/dL.

Chemical Methods

Hemoglobin is determined based on the iron content.

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Gasometric Methods

Principle: The test is based on the fact that one molecule of O_2 binds to each iron atom of hemoglobin. Thus, oxygen combining capacity indirectly measures the amount of Hb.

SUMMARY

- Estimation of hemoglobin is one of the important investigations used for the diagnosis of anemia.
- There are several methods of hemoglobin measurement. Standard method used is cyanmethemoglobin (HiCN) method which is based on the absorbance of cyanmethemoglobin at 540 nm.
- Automated analysers are available which measure not only hemoglobin but also various parameters like cell counts, PCV and absolute values (MCV, MCH and MCHC).

SELF-ASSESSMENT EXERCISES

I. Short Notes

1. Various methods of Hb estimation. Mention the advantages and disadvantages of each method
2. Advantages of cyanmethemoglobin method

II. Multiple Choice Questions

1. The following is NOT a method of hemoglobin estimation
   A. Cyanmethemoglobin method
   B. Sahli's method
   C. Gasometric method
   D. Wintrobe method

2. Best method for hemoglobin estimation is
   A. By Sahli's hemoglobinometer
   B. Cyanmethemoglobin method
   C. Alkaline hematin method
   D. Oxyhemoglobin method

ANSWERS

1. D **2.** B

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CHAPTER 41

Cell Count

CHAPTER OUTLINE

- Introduction
- Red Blood Cell Count
- Total WBC Count
- Platelet Count
- Absolute Eosinophil Count

INTRODUCTION

Red blood cells, white blood cells and platelets are the formed elements of blood. Their counts are altered in various diseases and the estimation of their counts is a basic hematologic investigation.

Hemogram (complete blood counts): It includes hemoglobin, total leukocyte count, RBC count, platelet count, differential leukocyte count, PCV, ESR and peripheral smear examination.

Equipment

Hemocytometer: The hemocytometer (Fig. 41.1) is an instrument used for counting blood cells. The commonly used instrument is the improved Neubauer counting chamber.

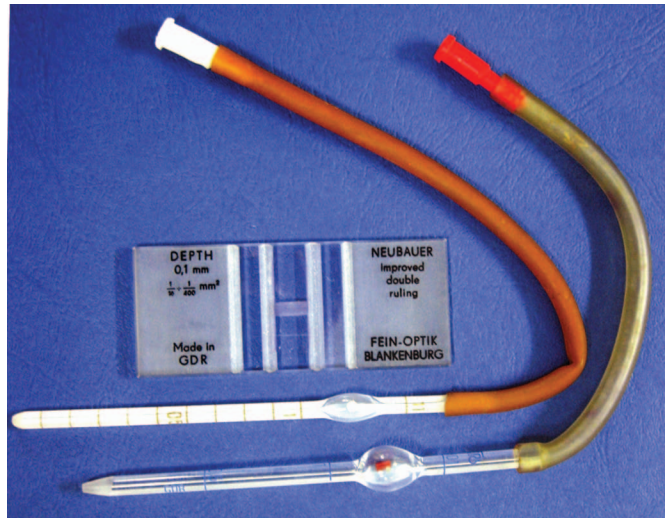


Fig. 41.1: Hemocytometer consisting of improved Neubauer counting chamber in the center with WBC (white bead) and RBC (red bead) pipette

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- **RBC pipette**
- **WBC pipette**

  Note: Unopettes are self-contained diluting device that can eliminate pipetting errors or manual errors and can be used instead of RBC and WBC pipettes.

  Uses of the (red/white) glass bead in the bulb of (RBC/WBC) pipette
  - **Identification** of pipette (red in RBC and white in WBC pipette).
  - Helps in **mixing blood** sample with diluting fluid.
  - Ensures that the **pipette is dry.** If pipette is wet, the corresponding bead will be sticking to the wall of the pipette.
- **Improved Neubauer counting chamber (Fig. 41.2):** WBC count is performed in the 4 corner squares and RBC count in the middle square.

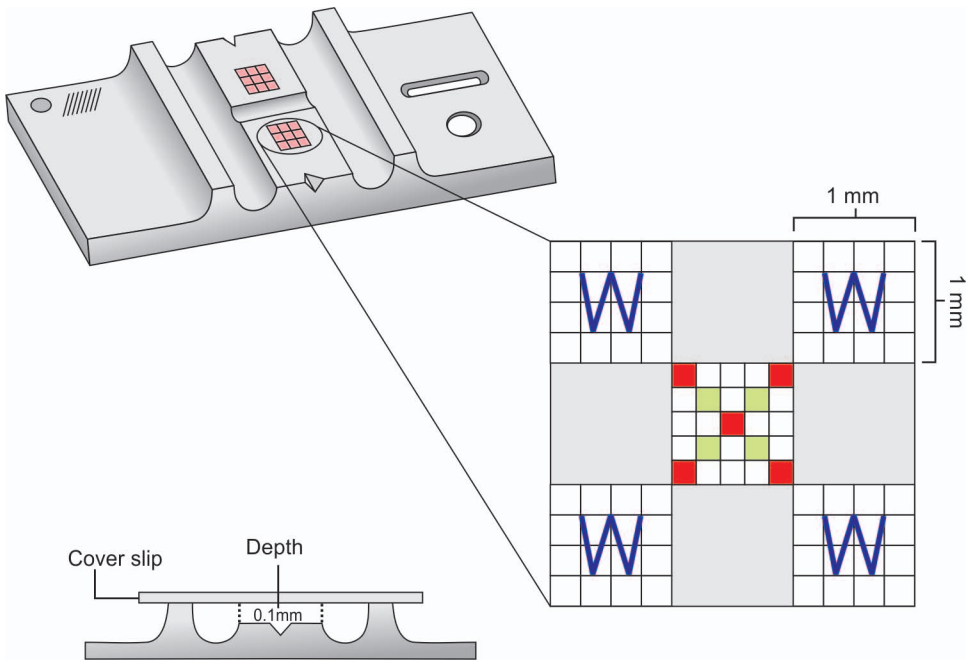


Fig. 41.2: Close-up view of improved Neubauer counting chamber. WBC counting areas marked W and RBC counting area in red square. The entire central square is used for platelet count

RED BLOOD CELL COUNT

It can be measured by manual method using an improved Neubauer counting chamber or by automated analyser.

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Manual Method

Equipment

- **Improved Neubauer counting chamber**
- **RBC** pipette (Thoma red cell pipette):
  - It consists of a graduated capillary tube divided into 10 parts marked 0.5 at the fifth mark and 1.0 at the tenth mark
  - The capillary tube opens into a mixing bulb containing a glass bead which facilitates mixing of the blood and the diluent.
  - There is another short capillary tube above the bulb marked 101.
  - Each pipette is provided with an aspirating tube.
  - In the red cell pipette, blood is drawn up to the 0.5 mark and diluent up to the 101 mark. The resulting dilution is 1:200.

Uses of the RBC pipette

- RBC count.
- Platelet count.
- WBC count when total count is very high.

While loading the counting chamber, discard the first 2-3 drops since it contains only diluting fluid.

Principle

The blood is diluted with an isotonic solution and the erythrocytes in a portion of the diluted fluid are counted in a hemocytometer under a microscope.

RBC Diluting Fluids

Composition of two types of RBC diluting fluids is shown in Tables 41.1 and 41.2.

Table 41.1: Composition of Hayem's fluid

Constituents	*Quantity*	*Action*
Mercuric chloride	0.5 gm	Prevents growth of bacteria
Sodium chloride	1.0 gm	Provides iso-osmolarity
Sodium sulfate	5.0 gm	
Distilled water	200 mL	

Table 41.2: Composition of Dacie's fluid (formal-citrate solution)

Constituents	*Quantity*	*Action*
Sodium citrate dihydrate	5.0 gm	Anticoagulant
Formalin	1 mL	Fixation of cells, retards bacterial and fungal growth
Distilled water	100 mL	

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Technique

- Gently mix the blood in the EDTA vial so that the cells mix well with plasma.
- Draw the **blood upto 0.5 mark** of RBC pipette. Wipe off the excess blood from sides of the tip of the pipette.
- Dip the tip of the pipette in the **RBC diluting fluid** and draw the fluid **upto mark 101.**
- Holding the pipette horizontally in its long axis, rotate it slowly to ensure **thorough mixing of blood and diluent.** This is facilitated by the red bead in the bulb.
- Place the cover slip on the ruled area of the chamber.
- **Discard the first 2 to 3 drops** (since the fluid has not mixed with blood) of RBC fluid from the pipette.
- **Charge the chamber** by placing the tip of the pipette just under the cover slip and allow the fluid to flow by capillary action till the counting chamber is just filled.
- Wait for 2-5 minutes for the cells to settle in the chamber.
- Examine the central ruled area for red cell counting by examining under low power objective.
- **Count RBCs** using high power objective **in the 5 red squares** and total them together.
- RBC count = counted cells × 10,000 per cu mm (mm^3) of blood.

Sources of error

- **Equipment:** Inaccurate graduation of pipette and Neubauer chamber.
- **Improper technique:** Improper blood collection or improper mixing of blood sample.
- **Human errors:** Improper charging of Neubauer chamber.

Other Methods for RBC Count

- **Sahli's hemoglobinometer pipette:** Take 0.02 mL of blood and add 4 mL of RBC fluid. The resulting dilution is 1 in 200.
- **Automated analyser.**

Normal Range for RBC Count (Table 41.3)

Table 41.3: Normal range of RBC count

Adult male	4.4-6.0 million/cu mm (4.4-6.0 × 10^{12}/L) (4.4-6.0 × 10^6/µL)
Adult female	3.8-5.0 million/cu mm (3.8-5.0 × 10^{12}/L) (3.8-5.0 × 10^6/µL)
At birth	6.0-7.0 million/cu mm (6.0-7.0 × 10^{12}/L) (6.0-7.0 × 10^6/µL)

Note: RBC count is reported as red cells per cubic millimeter (cu mm/mm^3) or 10^{12}/L or 10^6/µL of blood.

Causes of Increased RBC Count

The common causes of increased RBC count are shown in Table 18.1.

Causes of Decreased RBC Count

- Anemias
- Hemodilution

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TOTAL WBC COUNT

Total leukocytes/WBC count (TLC) in the blood can be measured either by manual method using an improved Neubauer counting chamber or by automated analyser.

Manual Method

Equipment

- **Improved Neubauer counting chamber**
- **WBC pipette**
  - The size of the pipette is smaller than that of RBC pipette and it has a white glass bead in the bulb which facilitates mixing of the blood and the diluent.
  - It has 3 markings of 0.5, 1 and 11.
  - In the white cell pipette, blood is drawn up to the 0.5 mark and diluent up to the 11.0 mark.
  - The dilution in this case is 1:20.

Uses of the WBC pipette

- Manual WBC count
- Absolute eosinophil count (AEC)
- Sperm count
- Any fluid (e.g. synovial fluid) cell count.

Principle

Whole blood is diluted with WBC fluid and the glacial acetic acid in the fluid lyses the red cells. The nuclei of white blood cells are stained by gentian violet, which are counted in the WBC areas in the improved Neubauer counting chamber.

WBC Diluting Fluid

Composition of WBC diluting fluid is shown in Table 41.4.

Table 41.4: Composition of WBC diluting fluid (Turk's Fluid)

Constituents	*Quantity*	*Action*
Glacial acetic acid	2 mL	Lyses the RBCs
1% Gentian violet	5 drops	Stains nuclei of WBC
Add water	100 mL	Diluent

Technique

- Gently mix the blood in the EDTA vial, so that the cells mix well with plasma.
- Draw the **blood in the WBC pipette upto 0.5 mark.** Wipe off the excess blood from sides of the tip of the pipette.
- Dip the tip of the pipette in the **Turk's fluid** and draw the fluid **upto mark 11.**
- Holding the pipette horizontally in its long axis, rotate it slowly to ensure thorough mixing of blood and diluent. This is facilitated by the white bead in the bulb.

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- Place the cover slip on the ruled area of the chamber.
- Discard the first 2 to 3 drops (since the fluid has not mixed with blood) of WBC fluid from the pipette.
- **Charge the chamber** by placing the tip of the pipette just under the cover slip and allow the fluid to flow by capillary action till the counting chamber is just filled.
- Wait for 2-3 minutes for the cells to settle in the chamber.
- Examine the ruled WBC areas under the microscope using a low power objective. White cells are identified because of their nuclei stained by gentian violet present in the Turk's fluid.
- Count the total number of WBCs in the areas marked in the Fig. 41.2 (in big squares, each area is divided into 16 medium-sized squares).
- WBC count = counted cells × 50/cu mm of blood.
- The result thus obtained is expressed as:
  Total WBC = per cu mm (mm^3) of blood.

Sources of Error

- **Equipment:** Inaccurate graduation of pipette and Neubauer chamber.
- **Improper technique**: Improper blood collection or improper mixing of blood sample.
- **Human errors**: Improper charging of Neubauer chamber.

Note:

- When there is marked leukopenia, it is advisable to use 1 in 10 dilutions for greater accuracy by taking blood up to mark 1 instead of 0.5.
- In cases of very high WBC counts, a dilution of 1 in 100 or 1 in 200 using RBC pipette may be required for accurate count.
- If nucleated red cells (nRBC) are present correction of WBC count should be done.

Corrections of Total WBC Count for nRBC

Nucleated red cells are not lyzed and counted with WBCs. If nucleated red blood cells (nRBCs) are 5 or more per 100 WBCs in the differential leukocyte count, a correction for nRBCs should be made.

$$\text{Corrected TLC/ cu mm} = \frac{\text{Uncorrected TLC (per cu mm)} \times 100}{\text{No. of nRBCs per 100 WBCs} + 100}$$

Other Methods for WBC Count

- **Sahli's hemoglobinometer pipette:** Take 0.4 mL of Turk's fluid in a small test tube and add 0.02 mL of blood into it using the hemoglobin pipette. The resulting dilution is 1 in 20. Charge the improved Neubauer counting chamber with a dropper.
- **Automated analyser.**

Uses

- Support diagnosis of various infections and inflammatory lesions.
- Diagnosis of myeloproliferative neoplasms, myelodysplastic syndromes, leukemias and various other hematological disorders.

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Normal Range for WBC Count (Table 41.5)

Table 41.5: Normal range of WBC count	
Adults	4000-11000 cells/cu mm (4-11 × 10^9/L) (4-11 × 10^3/μL)
At birth	8000-22000 cells/cu mm (8-22 × 10^9/L) (8-22 × 10^3/μL)
Childhood	6000-15000 cells/cu mm (6-15 × 10^9 /L) (6-15 × 10^3/μL)

Note: Total WBC count is reported as white blood cells per/cubic millimeter (cu mm/mm^3)/ or 10^9/L or 10^3/μL of blood.

Increased WBC Count

Definition: Total leukocyte counts more than 11,000/cu mm (11 × 10^9/L) is termed as leukocytosis.

Causes: Common causes of leukocytosis are shown in Table 41.6.

Table 41.6: Common causes of leukocytosis
• Infections ▪ Bacterial ▪ Viral infections (e.g. infectious mononucleosis) • Leukemia ▪ Acute ▪ Chronic: Chronic lymphocytic leukemia and chronic myeloid leukemia • Leukemoid reactions • Physiological ▪ Pregnancy ▪ Exercise

Decreased WBC Count

Definition: Total leukocyte count less than 4,000/cu mm (4 × 10^9/L) is termed as leukopenia.

Causes: Common causes of leukopenia are shown in Table 41.7.

Table 41.7: Common causes of leukopenia
• Typhoid and paratyphoid • Anemia ▪ Aplastic anemia ▪ Megaloblastic anemia • Hypersplenism • Drugs including cytotoxic drugs • Radiation • Rarely leukemia

Neutophilia and neutropenia are discussed under Chapter 19 (Tables 19.3 and 19.5).

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PLATELET COUNT

Manual Method

Equipment

- Improved Neubauer counting chamber
- WBC pipette

Principle

Anticoagulated blood is diluted with a diluent that lyses the red cells (e.g. 1% ammonium oxalate) leaving visible platelets. Dacie's fluid and Rees-Ecker fluid can also be used for RBC count.

Platelet Diluting Fluids

- **Dacie's fluid** (Table 41.8).

Table 41.8: Composition of Dacie's fluid

Constituents	*Quantity*	*Actions*
Sodium citrate dihydrate	5 gm	Prevents coagulation
Formalin	1 mL	Fixation of cells, retards bacterial and fungal growth
0.2% brilliant cresyl blue	0.1 mL	Stains platelets
Distilled water	100 mL	

The above fluid is used as a stock solution, which is diluted 1 in 10 before use

- **Rees-Ecker fluid** (Table 41.9).

Table 41.9: Composition of Rees-Ecker fluid

Constituents	*Quantity*	*Actions*
Sodium citrate	3.8 gm	Prevents coagulation
Brilliant cresyl blue	50 mg	Stains platelets
Formalin	0.2 mL	Fixation of cells, retards bacterial and fungal growth
Distilled water to make	100 mL	

Platelets are stained pale blue by brilliant cresyl blue

- **Brecher-Cronkite fluid:** It consists of 1% ammonium oxalate solution which is filtered and stored at 4°C.

Technique

- Gently mix the blood in the EDTA vial, so that the cells mix well with plasma.
- Draw the **blood in the WBC pipette up to 0.5 mark.** Wipe off the excess blood from sides of the tip of the pipette.

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- Dip the tip of the pipette in 1% ammonium oxalate (Brecher-Cronkite fluid) and draw the **fluid up to mark 11.**
- Holding the pipette horizontally in its long axis, rotate it slowly to ensure thorough mixing of blood and diluent. This is facilitated by the white bead in the bulb.
- Place the cover slip on the ruled area of the chamber.
- Discard the first 2 to 3 drops (since the fluid has not mixed with blood) of fluid from the pipette.
- **Charge the chamber** by placing the tip of the pipette just under the cover slip and allow the fluid to flow by capillary action till the counting chamber is just filled.
- Keep the charged chamber in a petri dish with wet filter paper at the bottom for 5 minutes for the platelets to settle down.
- Platelets are counted in the entire central 1 × 1 mm square.
- Platelets appear as highly refractile small structures. They are visualized easily with the following adjustments of the microscope:
  - Reducing the illumination by closure of the iris diaphragm
  - Lowering the condenser
  - Carefully altering the focus with fine adjustment.
- Platelet count = Total counted platelets in the central square × 200 per cu mm of blood.

Sources of Error

- If platelets are agglutinated in the chamber, do not carry out the platelet count.
- Always compare the count with the peripheral smear.
- Other errors are similar to that for RBC and WBC count.

Automated Analyser

Platelet count can also be obtained by electronic particle counters.

Normal Range

1,50,000 to 4, 50,000 platelets per cu mm (150- 450 × 10^9/L) (150-450 × 10^3/μL)

Note: Platelet count is reported as number of platelets per cubic millimeter (cu mm/mm^3) or 10^9/L or × 10^3/μL of blood.

Decreased Platelet Count

Definition: Thrombocytopenia is the term used when the platelet count is less than 1, 50,000/cu mm (150 × 10^9/L).

Causes: Discussed under Chapter 35.

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Increased Platelet Count

Definition: Platelet count more than 4, 50,000/cu mm (450×10^9/L) is known as thrombocytosis.

Causes: Various causes of thrombocytosis are shown in Table 41.10.

Table 41.10: Causes of thrombocytosis

Idiopathic/primary (autonomous production)
- Essential thrombocytosis
- Polycythemia vera
- Chronic myeloid leukemia

Secondary (reactive thrombocytosis)
- Iron deficiency
- Malignancy
- Following hemorrhage
- Following splenectomy

ABSOLUTE EOSINOPHIL COUNT

Manual Method

Equipment

- Improved Neubauer counting chamber
- WBC pipette.

Diluting Fluid

Dunger's fluid composition is shows in Table 41.11. It should be preserved in the refrigerator.

Table 41.11: Composition of Dunger's fluid

Constituents	*Quantity*	*Action*
Eosin	200 mg	Stains the eosinophil granules orange red
Acetone	10 mL	Fixation of eosinophils
Water	80 mL	Lysis of red cells and white cells (except eosinophils which are resistant)

Technique

- Gently mix the blood in the EDTA vial, so that the cells mix well with plasma.
- Draw the **blood in the WBC pipette upto mark 1.** Wipe off the excess blood from sides of the tip of the pipette.
- Dip the tip of the pipette in the **Dunger's fluid** and draw the fluid **upto mark 11.** The dilution is 1 in 10.
- Holding the pipette horizontally in its long axis, rotate it slowly to ensure thorough mixing of blood and diluent. This is facilitated by the white bead in the bulb.

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- Place the cover slip on the cleaned ruled area of the counting chamber.
- Discard the first 2 to 3 drops (since the fluid has not mixed with blood) of WBC fluid from the pipette.
- **Charge the chamber** by placing the tip of the pipette just under the cover slip and fluid flows under it by capillary action. Allow till the counting chamber is just filled.
- Wait for 5 minutes for the eosinophils to settle in the chamber.
- **Count the number of eosinophils in the 4 corner squares** under the microscope using a low power objective. Eosinophils are identified because of their bright red granules and count should be done within 30 minutes.
  Absolute eosinophil count (AEC) = Total number of eosinophils in 4 squares × 25.

AEC using Fuch's Rosenthal chamber: It is preferable to do AEC using Fuch's Rosenthal chamber, where the depth of chamber is 0.2 mm compared to 0.1mm in improved Neubauer chamber. This increase in depth means more cells will be counted thereby increasing the accuracy of AEC.

Normal Range of Eosinophil Count

Eosinophils constitute 1 to 6% of circulating WBCs. The range of absolute eosinophil count is 40-450 cells/ cu mm ($0.04\text{-}0.45 \times 10^9$/L).

Use

AEC in blood is useful in diagnosis, treatment and follow-up of cases of eosinophilia.

High AEC (Eosinophilia)

Definition: Eosinophilia is defined as an absolute eosinophil count of more than 450/cu mm (mm^3).
Causes: Refer Table 19.6.

Automated Analyser/Cell Counters

These have presently replaced the manual methods.

SUMMARY

- Red cells, white cells and platelets are the three formed elements of blood. Estimation of their counts is an essential hematologic investigation which may be performed by manual methods or by automated analysers.
- The improved Neubauer chamber, RBC pipette, WBC pipette and diluting fluids are used for manual counts of any cell.
- Automated analysers have now replaced these manual methods and the counts are comparatively more accurate.

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SELF-ASSESSMENT EXERCISES

I. Short Notes

1. Uses of WBC pipette and method of WBC count
2. Uses of RBC pipette and method of RBC count
3. Causes of increased RBC count
4. RBC diluting fluid
5. WBC diluting fluid
6. Platelet diluting fluid and method of platelet count
7. Absolute eosinophil count
8. Leukopenia
9. Thrombocytosis and its causes

II. Multiple Choice Questions

1. **The diluting fluid used for RBC count is**
   A. Turk's fluid
   B. Hayem's fluid
   C. Dunger's fluid
   D. Rees-Ecker fluid
2. **The diluting fluid used for WBC count is**
   A. Turk's fluid
   B. Hayem's fluid
   C. Rees-Ecker fluid
   D. Dacie's fluid
3. **All the following diluting fluids can be used for Platelet count EXCEPT**
   A. Brecher-Cronkite fluid
   B. Dacie's fluid
   C. Dunger's fluid
   D. Rees-Ecker fluid
4. **The diluting fluid used for absolute eosinophil count is**
   A. Turk's fluid
   B. Hayem's fluid
   C. Dunger's fluid
   D. Rees-Ecker fluid

ANSWERS

1. B **2.** A **3.** C **4.** C

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Chapter 42

Peripheral Blood Smear Examination

Chapter Outline

- Introduction
- Stains for Blood Smear
- Preparation of the Peripheral Blood Smear
- Fixation of the Smear
- Staining of the Smear
- Examination of a Peripheral Blood Smear
- Red Blood Cells
- White Blood Cells
- Platelets
- Hemoparasites

INTRODUCTION

Peripheral smear (peripheral blood film) is the most important, valuable and frequently asked investigation in hematology laboratory. It provides the following information:

- **Red cell morphology:** Morphological features of RBCs are important for diagnosis of anemias and other hematologic disorders.
- **WBC disorders and differential leukocyte count** (DLC): Quantitative and qualitative changes in WBCs help in diagnosis of both hematologic and non-hematologic disorders.
- **Platelet number and morphology**: These features are useful in the diagnosis of bleeding disorders.
- **Cross check the CBC parameters:** It helps in cross-checking the complete blood count (CBC) parameters derived from automated cell counters.
- Detection of **blood parasites** (hemoparasites).

STAINS FOR BLOOD SMEAR

- The blood cells contain cellular structures which vary in their reaction (pH), some are acidic and others being basic.
- The aniline dyes used in staining blood smears are of two general classes: basic dyes such as methylene blue and acidic dyes such as eosin.
- All stains which are made of combinations of acidic and basic dyes are called **Romanowsky stains.** The differences between the various Romanowsky stains are mainly in the proportion of the reagents and in their preparation.

Romanowsky Stains

A mixture of methylene blue (basic stain) and eosin (acidic stain) was first prepared by Romanowsky in 1891 and employed on his work on malarial parasite. The action of these stains depends on compounds formed by the interaction of methylene blue and eosin. Methylene blue

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on oxidation produces colored compounds called azures that have the ability to combine with eosin. Oxidation is achieved during maturation/chemical treatment of the stain. The azures are responsible for different shades of staining in the smears.

- Nuclei and structures in the blood which are stained by the basic dyes are called basophilic.
- Structures that take up only acidic dyes are called acidophilic or eosinophilic.

Romanowsky group includes the following stains:

- ***Leishman stain*** which contains Leishman powder and acetone free methyl alcohol (methanol).
  - Preparation of stain: Dissolve 1 gm of Leishman powder in 500 ml of acetone free methyl alcohol in a conical flask. Warm it at 50^0 C for 15 minutes. Keep it for ripening by exposing to sunlight for 2 days. **Acetone free methyl alcohol** acts as a **fixative**. Since acetone destroys the cells, methyl alcohol should be free from acetone.
- ***Giemsa stain*** contains Giemsa stain powder, glycerol and acetone free methyl alcohol.
  - Preparation of stain: Dissolve 1 gm of Giemsa stain powder in 66 ml of glycerol at 60° C for 2 hours. After cooling add 66 ml of acetone free methyl alcohol and mix it. Keep it at 37° C for one week for ripening.
- ***Wright stain***
- ***Jenner stain***
- ***Jenner-Giemsa stain***

PREPARATION OF THE PERIPHERAL BLOOD SMEAR (FIGS 42.1A TO D)

- A drop of blood is placed on a clean grease free glass slide 1 cm away from one end.
- Take a spreader (either another slide or a narrower piece of slide with smooth edge) and place its smooth edge over the drop of blood so as to spread the blood along its edge.
- Place the slide at about 30° to 40° angle and make the smear with a smooth (not jerky) forward movement. The resulting ideal smear should be about 2.5 to 3.5 cm in length.
- Allow it to dry at room temperature and label.

Features of a Well-made Peripheral Smear

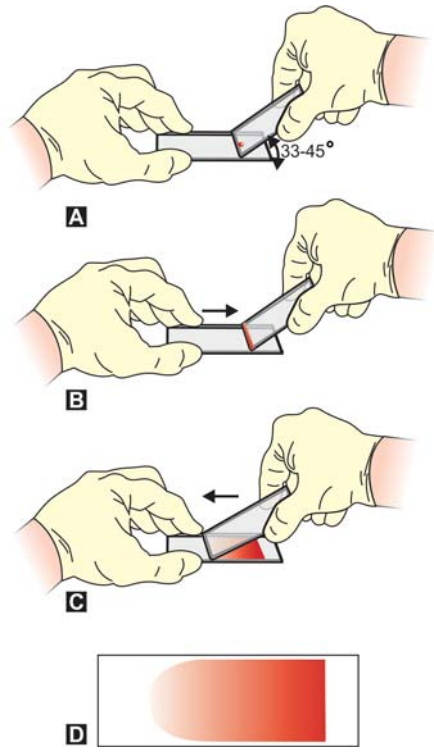
- Length: Smear should be 2/3 to 3/4 of the length of the slide.
- Shape: The smear should be either tongue or finger-shaped (smooth without any irregularities or holes).

Sources of Error

- Presence of grease on the slide causes blank oval areas in the smear.
- If spreader edge is not smooth, the tail of the smear shows ragged edges and most of the neutrophils accumulate in the tail end. The rest of the smear shows paucity of neutrophils, a process termed as tailing of the smear. This results in erroneous differential counts.

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Figs 42.1A to D: Different steps in the preparation of peripheral blood smear and appearance of a well-made smear

FIXATION OF THE SMEAR

- The blood smears should be fixed within 4 hours for good staining.
- Blood films are 'fixed' by **acetone-free methyl alcohol** which is present in most of the commonly used stains, namely Leishman or Wright or Giemsa stain.
- It can also be fixed by dipping the slide 8 to 10 times in a coplin jar with methyl alcohol.

STAINING OF THE SMEAR

Staining of the smears is carried out using *Romanowsky stains* which stain red cells, white cells, platelets and parasites.

Procedure of Staining with Leishman Stain

- Keep the peripheral smear on the staining rack.
- **Pour the undiluted stain** on the slide with a dropper so that stain covers the entire upper surface of slide.
- Wait for **2 minutes** for the methyl alcohol to **fix the smear**. Then pour double the amount of buffer water on the slide. Gently mix using a dropper.

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- Wait for another **8-10 minutes** (time depends on the batch of stain) for **staining**. Look for greenish metallic scum to appear.
- Displace the stain by pouring running water from one side of the slide so that the stain scum is drained off the slide and no more stain is left on the slide.
  - Precaution: Do not discard the stain from slide by just tilting it, since it will result in stain precipitates over the smear.
- Remove the slide from staining rack and keep it in slanting position to dry it up.
- Examine the slide under microscope.

Sources of Error

- Buffered pH of 6.8 is important for good quality of staining.
  - Acidic pH red cells appear pink and WBCs takes up very light stain.
  - Basic pH red cells appear blue and WBCs appear bluish-black.
- Staining errors:
  - If smear is overstained, wash the smears with undiluted Leishman stain or methanol for few seconds.
  - If smear is understained, restain by adding both Leishman stain and buffer for required period depending on the extent of understaining.

Procedure of Staining with Giemsa Stain

- Peripheral smear prepared is fixed by dipping 8 to 10 times in a coplin jar containing methanol.
- Allow the slide to dry.
- Pour the diluted stain (1 in 10) and wait for 20-30 minutes.
  Note: The amount of dilution and time for staining varies with each batch of stain.
- Wash the slide (as in staining by Leishman) and dry it.

EXAMINATION OF A PERIPHERAL BLOOD SMEAR

A well prepared peripheral smear usually has three parts namely, head, body and tail. The best area of the smear to study the morphology of cells is the body, where the cells are well separated out and touch one another without overlapping. Apply a thin layer of oil over the smear which makes the appearance of cells very clear.

First look under low power, followed by high power and oil immersion. Observe the following:

- The **quality of the film** and uniformity of staining.
- **Red blood cells:** Select an area (best between tail and body of the smear) suitable for examination of red cells in the thin area of the smear where the red cells are evenly placed and just touch each other. Look for number, distribution and staining of the red cells, degree of rouleaux formation if present.

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- **White blood cells:** Number and distribution of white cells
- **Platelets:** Number and distribution
- **Hemoparasites** (such as malaria, microfilaria)
- **Any abnormal cells.**

RED BLOOD CELLS

Red cells are examined for (1) size (2) shape (3) color (4) inclusions and (5) other abnormalities.

1. **Size:** In the peripheral smear, size of the red blood cells is compared with that of a small lymphocyte which has size of about 7-8 µm. The red cells are called microcytic if smaller than small lymphocyte and macrocytic if larger than 9 µm. Appearance of size variation and conditions responsible for these variations are shown in Fig. 42.2.

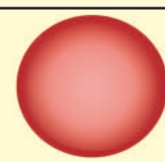
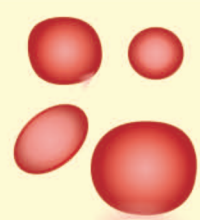
TERM	DESCRIPTION	APPEARANCE	CONDITION
Normocyte	• Normal RBC is biconcave, 7.2 µm in diameter		• Normal RBC • Normocytic normochromic anemia
Microcyte	• RBCs smaller than normal and less than 6 µm in diameter		• Iron deficiency anemia • Thalassemia
Macrocyte-round	• Larger than normal and more than 9 µm in diameter		• Liver disease • Alcohol abuse
Macro-ovalocyte	• Larger than normal, more than 9 µm in diameter and also oval		• Megaloblastic anemia due to vitamin B_{12} and folic acid deficiency
Anisocytosis	• Variation in size		• Severe anemia of any type

Fig. 42.2: Variation in size of red blood cells and associated conditions

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2. Shape: Variation in red cell shape and conditions responsible for these variations are shown in Figs 42.3 A and B.

TERM	DESCRIPTION	APPEARANCE	CONDITION
Normal	• Biconcave, flattened on smear		• Normal
Poikilocytosis	• Variation in shape		• Severe anemia
Spherocytes	• Smaller and stain darker without any central pallor		• Hereditary spherocytosis
Target cells or leptocytes	• Only the periphery and the central regions of the cell appear hemoglobinized		• Thalassemia • Sickle cell anemia • Hemolytic anemia • Postsplenectomy • Liver disease
Sickle cells	• Thin, elongated, slightly curved and have shape of a sickle		• Sickle cell anemia
Bite cells	• Pairs of spicules		• G6PD deficiency
Schistocytes (fragmented cells)	• Small, irregular shaped, triangular or spiculated cells		• Microangiopathic hemolytic anemia (e.g. DIC, TTP)

Fig. 42.3A

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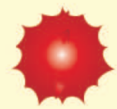
TERM	DESCRIPTION	APPEARANCE	CONDITION
Acanthocytes	• RBCs with few spicules of uneven (irregular) length and shape		• Abetalipoproteinemia • Liver disease
Echinocyte/ Burr cells	• Very small irregular shrunken cells with pointed projections. Resemblance to the small thorny 'burrs'		• Uremia
Elliptocyte or Ovalocyte	• Oval in shape		• Hereditary ovalocytosis • Hemolytic anemia
Pencil shaped cells	• Elongated thin cells (exaggerated ovalocytes)		• Iron deficiency anemia
Tear drop and pear-shaped cells	• These abnormal RBCs have a tear drop or pear-like shape		• Myelofibrosis • Marrow infiltration
Stomatocytes	• Red cells with a slit like area of central pallor		• Hereditary stomatocytosis • Liver disease

Fig. 42.3B

Figs 42.3A and B: Variation in shape of red blood cells and associated conditions

3. **Color:** Variations in red cell color are depicted in Fig. 42.4.
   - Sometimes red cells appear to be hyperchromic without a central area of pallor. This is not actually due to an increase in the concentration of hemoglobin in the cell, but due to change in cell shape and increase in the thickness of the cell membrane in macrocytes.

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TERM	DESCRIPTION	APPEARANCE	CONDITION
Normochromic	Cell with central 1/3 pallor		• Normal RBC
Hypochromic	Central pallor more than 1/3		• Iron deficiency anemia • Thalassemia
Polychromasia	Cells stained purple and are larger than normal		• Hemolytic anemias • Response to therapy in deficiency anemias

Fig. 42.4: Variation in color of red blood cells

4. **Inclusions in the red cells:** Inclusions in red blood cells and associated conditions are presented in Fig. 42.5.
5. **Other abnormalities:** Example, rouleaux formation (Fig. 42.6).

WHITE BLOOD CELLS

White cells (leukocytes) are examined for the following features:

- **Number:** Note whether the number is decreased, normal or increased.
  - Assessment of approximate total white cell count: Observe at least 10 high power fields in the thick portion of the smear where red blood cells overlap. Estimate the approximate number of WBCs/high power filed. Each WBC/high power field is approximately equivalent to 2,000 WBC/cu mm.
- **Abnormal or immature forms**
- **Predominant cell type:** This is determined by performing the differential white cell count. At least 100 white cells should be counted. Normal range of differential leukocyte count is shown in Table 42.1.

Table 42.1: Normal range of different leukocyte count (DLC)

Type of white blood cells	*Normal range*
Granulocytes	
• Neutrophil (polymorphonuclear leukocyte)	40-70% (2.0-7.0 × 10^9/L)
• Eosinophil	1-6% (0.02-0.5 × 10^9/L)
• Basophil	0-1% (0.02-0.1 × 10^9/L)
Lymphocytes	20-40% (1.0-3.0 × 10^9/L)
Monocyte	2-10% (0.2-1.0 × 10^9/L)

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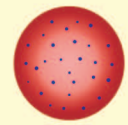
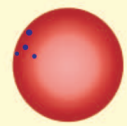
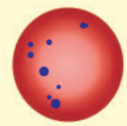
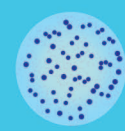
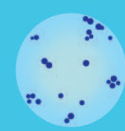
TERM	DESCRIPTION	APPEARANCE	CONDITION
Basophilic stippling (punctate basophilia)	• Red cells contain small, blue black inclusions and represent precipitated ribosomal RNA		• Lead poisoning • Megaloblastic anemias • Hemolytic anemias like thalassemias • Sideroblastic anemias • Alcoholism
Howell-Jolly bodies	• Small, round, densely staining dark blue particles and represent nuclear remnants		• Megaloblastic anemias • Acute hemolytic anemias • Postsplenectomy state
Cabot rings	• Rings or figure of eight shaped and are probably remnants of the nuclear membrane		• Hemolytic anemia • Megaloblastic anemia • Leukemia • Rarely after splenectomy
Pappenheimer bodies/siderotic granules (RBCs with Pappenheimer bodies are called siderocytes)	• Aggregates of ferritin and appear pale blue, but are easily demonstrable with perl's stain (Prussian blue reaction)		• Sideroblastic anemias • Megaloblastic anemias • Hemolytic anemias • Postsplenectomy
Hemoglobin H inclusions	• Free β chains in red cells. Small, regular, multiple and diffusely distributed (golf ball like appearance). Stained by supravital stains		• α-thalassemias
Heinz bodies	• Refractive, single or multiple rounded inclusions of denatured globin detected by supravital stains		• Intake/exposure to oxidizing drugs or chemicals • G6PD deficiency • Unstable hemoglobin disease
Hemoglobin C crystals	• Crystallization of hemoglobin C		• Splenectomy in homozygous hemoglobin C

Fig. 42.5: Inclusions in red blood cells and associated conditions

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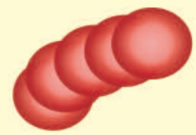
TERM	DESCRIPTION	APPEARANCE	CONDITION
Rouleaux formation	• Red cells aligned in aggregates (resembles a stack of coins)		• Seen when γ-globulin is increased and ESR is high • Multiple myeloma • Kala-azar • Chronic inflammatory diseases

Fig. 42.6: Rouleaux formation and associated conditions

Leukoerythroblastic Reaction or Blood Picture (Table 42.2)

Definition: The presence of normoblasts and immature cells of the neutrophilic series in the blood is known as a leukoerythroblastic reaction.

Table 42.2: Causes of leukoerythroblastic reaction or blood picture

Myelofibrosis with myeloid metaplasia
Leukemias (e.g. acute myeloid leukemia, acute lymphoid leukemia)
Storage diseases: Gaucher disease
Metastatic carcinoma

PLATELETS

They are examined for the following features under oil immersion. A single platelet under oil immersion is equivalent to 15,000 to 20,000 platelets/cu mm.

- **Number:** Normal/ increased/decreased
- **Form:** Note the size and shape.
- **Aggregation:** Smears made from finger-prick show aggregation of platelets or platelet clumps.

HEMOPARASITES

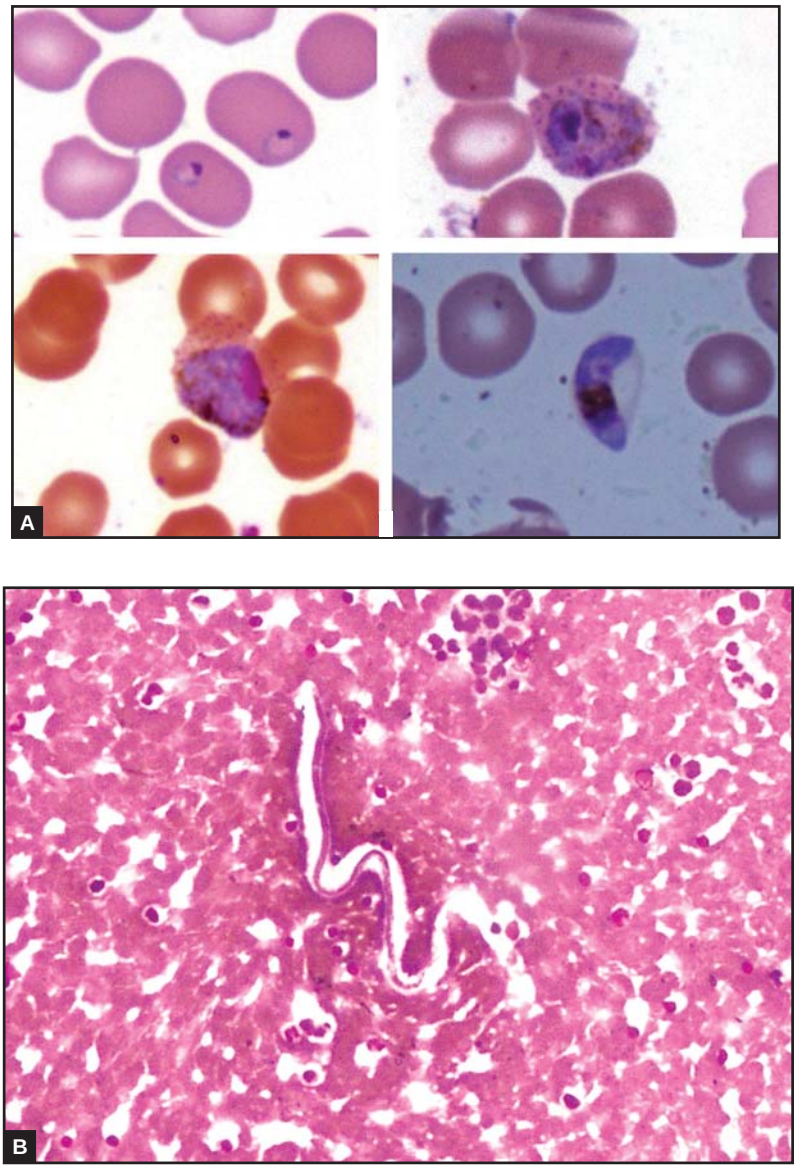
These include (1) malarial parasites, (2) microfilaria, (3) trypanosomes, (4) Leishmania Donovani and (5) babesiosis

1. **Malaria:** Malarial parasites can be demonstrated in the peripheral smear; most common are plasmodium vivax and falciparum.

   Thick Film for Malarial Parasite: Thick film method is preferred for mass surveys, quick diagnosis and when malarial parasites are likely to be few. It is advisable to make both thin and thick films on the same slide. Thick blood films can be stained by **Field's rapid method** or **Jaswant-Singh-Bhattacherji (JSB) stain.**

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Figs 42.7A and B: Plasmodium vivax ring (A upper), schizont (A left lower) and falciparum gametocyte (A right lower) and microfilaria (B)

Malarial Pigment: In malarial infections the globin of the hemoglobin is broken down and resynthesized into parasite protein. The unutilized part is hemozoin which is brown in color and known as malarial pigment. Hemozoin is responsible for the pigmentation of various organs like liver and spleen in malaria.

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Natural Protection Against Malaria

- Patients with glucose-6-phosphate dehydrogenase deficiency are protected against malaria. This is because malarial parasites need G6PD enzyme for survival.
- In patients with sickle cell disease, malarial parasites induce rapid sickling. The sickled red cells with parasite are sequestered by the reticuloendothelial cells of the spleen. This results in protection against malarial infection.

Plasmodium vivax: In the peripheral blood the trophozoites, schizonts and rarely gametocytes of plasmodium vivax may be found (Fig. 42.7A).

Plasmodium falciparum: In falciparum infections usually only ring stage and gametocytes may be demonstrated (Fig. 42.7B).

2. **Filariasis:** *Microfilaria* can also be demonstrated in peripheral blood (Fig. 42.7B).
3. **Trypanosomes:** These are motile flagellate protozoa.
4. **Leishmania Donovani:** It causes **kala-azar,** amastigote forms known as LD bodies can be found in the reticuloendothelial cells of the bone marrow, spleen and buffy coat preparations of peripheral blood. LD bodies are small, round, 2 to 4 μm in diameter with a nucleus and a rod shaped kinetoplast.
5. **Babesiosis:** It is a malaria like parasitic disease caused by babesia.

Reporting of Peripheral Smear: Format for peripheral smear reporting is shown in Table 42.3.

Table 42.3: Format for reporting peripheral blood smear

1. **RBCs: Comment on the following features**
   - Size: Normocytic (normal), microcytic or macrocytic (predominant feature to be mentioned)
   - Chromasia: Normochromic (normal), hypochromic, absence of central pallor
   - Anisocytosis: Present (slight /marked) or absent
   - Poikilocytosis: Present (slight /marked) or absent
   - Other abnormalities: Spherocytosis, sickle cells, target cells
   - Polychromatophilia: Present or absent
   - Nucleated red cells: Normoblasts- per 100 leukocytes
2. **WBCs: Comment on total WBC count as normal/increased /decreased**
   - **Differential Count (on 100 cells)**
   - Type of WBC (percentage)
     - Neutrophils
     - Eosinophils
     - Basophils
     - Lymphocytes
     - Monocytes
     - Immature leukocytes: Specify different cells
3. **Platelets:** Approximate judgment on adequate/increased /reduced
4. **Hemoparasites**

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SUMMARY

- Peripheral blood smear examination is the most important and valuable investigation which is done in a well-prepared and well stained smear. Smears are stained by Romanowsky stains, most commonly Leishman and Giemsa stains.
- Examination of peripheral blood smear in a systematic manner gives a lot of information in both hematological and non-hematological diseases.
- In the RBC series, examine size, shape and color. In the WBC series look for number and distribution of white cells. Examine platelets for number and distribution. Search for any hemoparasites (e.g. malaria, microfilaria) or abnormal cells.

SELF-ASSESSMENT EXERCISES

I. Short Notes

1. Importance of peripheral smear examination
2. Romanowsky stains
3. Hemoparasites
4. Leukoerythroblastic reaction

II. Multiple Choice Questions

1. **All of the following are Romanowsky stains EXCEPT**
   A. Leishman stain
   B. Giemsa stain
   C. Wright stain
   D. Hematoxylin stain
2. **The fixative present in Leishman stain is**
   A. Buffered formalin
   B. Acetone free methanol
   C. 10% formalin
   D. Ethyl alcohol
3. **In Leishman staining procedure, after the undiluted stain is added to the entire upper surface of smear, waiting for 2 minutes before pouring buffered water is**
   A. For fixation of smear
   B. For staining of smear
   C. To clean the background plasma
   D. For smear to stain uniformly
4. **Macro-ovalocytes are seen in**
   A. Megaloblastic anemia
   B. Sideroblastic anemia
   C. Aplastic anemia
   D. Hemolytic anemia

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5. **The most common cause of macrocytosis is**
   A. Iron deficiency
   B. Alcoholism
   C. Hemolytic anemia
   D. Pernicious anemia
6. **The feature typical of nonmegaloblastic type of macrocytic anemia is**
   A. Macro-ovalocyte
   B. Round macrocyte
   C. Howell-Jolly bodies
   D. Hypersegmented PMN
7. **Bite cells are seen in**
   A. G6PD deficiency
   B. Paroxysmal nocturnal hemoglobinuria
   C. Microangiopathic hemolytic anemia
   D. Hemolytic disease of newborn
8. **The type of RBC seen in abetalipoproteinemia is**
   A. Sickle cells
   B. Acanthocytes
   C. Echinocytes
   D. Schistocytes
9. **Shrunken red cells with pointed projections over cell surface are called as**
   A. Sickle cells
   B. Acanthocytes
   C. Echinocytes
   D. Schistocytes
10. **Schistocytes are**
    A. RBCs with malarial parasite
    B. RBCs with schizonts
    C. Fragmented RBCs
    D. Smaller RBCs without central pallor
11. **Tear drop cells are seen in**
    A. Myelofibrosis
    B. G6PD deficiency
    C. Immune thrombocytopenic purpura
    D. Chronic myeloid leukemia
12. **Basophilic stippling of red blood cells is a characteristic feature of**
    A. Lead poisoning
    B. Acute myeloid leukemia
    C. Chronic myeloid leukemia
    D. Idiopathic thrombocytopenic purpura
13. **Howell-Jolly bodies are seen in**
    A. Megaloblastic anemia
    B. Septicemia
    C. Tuberculosis
    D. Hemophilia

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14. Cabot rings may be seen in
A. Megaloblastic anemia
B. Iron deficiency anemia
C. Leukemoid reaction
D. Immune thrombocytopenic purpura

15. Hemoglobin H inclusions are seen in
A. α-thalassemia
B. β-thalassemia
C. Sickle cell anemia
D. G6PD deficiency

16. All of the following can be seen in the peripheral blood smear EXCEPT
A. Malarial parasites
B. Microfilaria
C. Leishmania donovani
D. Strongyloides stercoralis

ANSWERS

1. D	**2.** B	**3.** A	**4.** A	**5.** B	**6.** B	**7.** A	**8.** B
9. C	**10.** C	**11.** A	**12.** A	**13.** A	**14.** A	**15.** A	**16.** D

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CHAPTER 43 Reticulocyte Count

CHAPTER OUTLINE

- Methods of Reticulocyte Count
- Significance of Reticulocyte Count

Reticulocytes are immature, non-nucleated RBCs released from bone marrow. They are slightly larger than the mature RBCs.

METHODS OF RETICULOCYTE COUNT

- Visual method
- Automated method.

Visual Method

Staining in living state is known as **supravital staining.** Reticulocytes contain ribosomes and RNA, which can be stained by supravital stains like brilliant cresyl blue and new methylene blue in the live and unfixed state. These stains demonstrate blue filamentous or granular material (RNA and ribosomes). Reticulocytes stain ***polychromatic*** with Romanowsky stains and hence the term ***"polychromatophil"*** is used to indicate their presence in peripheral smear (Table 43.1).

Table 43.1: Composition of supravital stain for reticulocyte count

Contents	*Quantity*	*Action*
Brilliant cresyl blue/new methylene blue	1 gm	Stains reticulocyte RNA and ribosomes
3% sodium citrate solution	20 mL	Anticoagulant
0.9% sodium chloride solution	80 mL	Isotonic saline

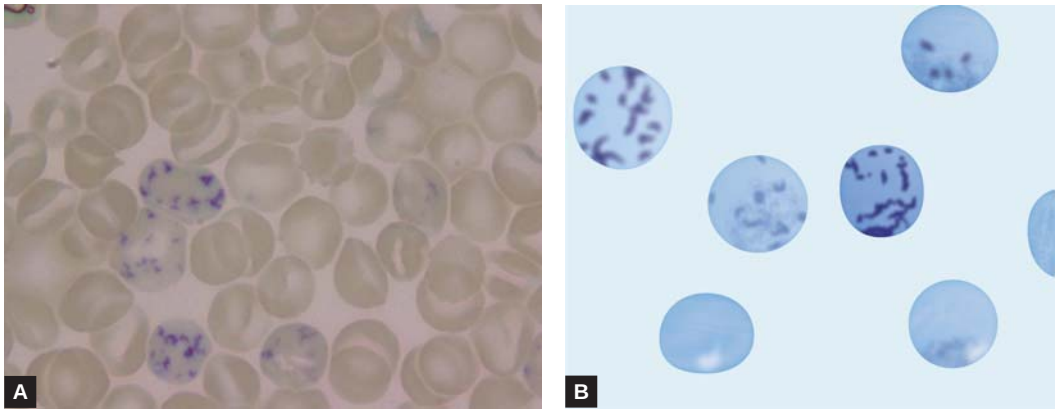
Brilliant Cresyl Blue/New Methylene Blue Stain (Figs 43.1A and B)

Procedure

- Take two drops each of supravital stain and blood in a test tube and mix.
- Incubate for 15 to 30 minutes at room temperature.

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Figs 43.1A and B: Smear shows reticulocyte with blue filamentous/granular material (new methylene blue stain)

- Take a drop of mixture on the slide and prepare the smear.
- Air-dry the smear.
- At least 1000 red cells are counted and percentage of reticulocytes is calculated.

Normal reticulocyte count: Reticulocyte count is expressed as percentage of total red cells.

- Normal 0.5-2.5%
- Newborn (cord blood) 1.2-4.0%

Reticulocyte Count Correction for Anemia

- In patients with anemia, the reticulocyte count does not reflect the true marrow response. So, the reticulocyte count obtained must be corrected for the severity of the anemia. The corrected reticulocyte count is calculated by multiplying the reticulocyte percentage by a factor adjusting for the degree of anemia (i.e. actual PCV divided by normal PCV for the age).

$$\textbf{Corrected Reticulocyte count} = \textbf{Reticulocyte count} \times \frac{\textbf{PCV}}{\textbf{Normal PCV for the age}}$$

- **Reticulocyte production index (RPI):** Early-released reticulocytes enter the circulation a day earlier than normal reticulocytes and the corrected reticulocyte count is further multiplied by 0.5 to obtain the reticulocyte production index (RPI).

$$\textbf{Reticulocyte index} = \textbf{Reticulocyte count} \times \frac{\textbf{PCV}}{\textbf{Normal PCV for the age}} \times 0.5$$

  - If RPI is greater than 2%; it indicates adequate erythropoietic response of the bone marrow and suggests that anemia is either due to acute blood loss or hemolysis.
  - If RPI is less than 2%; it indicates that the anemia is due to defective bone marrow production.

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Automated Method of Reticulocyte Count

Reticulocyte count can also be performed in automated cell counters or by a flow cytometer. It is superior and accurate to manual count. However, Howell-Jolly bodies, Heinz bodies, Pappenheimer bodies and giant platelets, when present may also be counted as reticulocytes.

SIGNIFICANCE OF RETICULOCYTE COUNT

Increased Reticulocyte Count

Reticulocyte count reflects the erythropoietic activity of the bone marrow and provides an estimate of red cell production. Increased eythropoiesis results in increased reticulocyte release.

Causes

- Hemolytic anemias
- Hemolytic crisis
- Hemorrhage
- Following treatment in iron/folic acid/vitamin B_{12} deficiency anemias. Highest counts are found on 6th/ 7th day of treatment and indicate marrow response to hematinics.

Reduced Reticulocyte Count

Reticulocyte count is reduced when there is decreased erythropoietic activity.

Causes

- Aplastic anemia
- Aplastic crisis due to parvo virus (hereditary spherocytosis and sickle cell disease).
- Pure red cell aplasia
- Fanconi anemia
- Myelofibrosis

Other Uses of Supravital Stains

HbH inclusions: Reticulocyte preparation can also demonstrate HbH inclusions (see Fig. 42.5) which represent free β chains and are seen in alpha-thalassemia. They appear as regular, tiny, multiple inclusions in the RBCs giving a **golf-ball like** appearance.

Heinz bodies: These are aggregates of denatured hemoglobin and appear as single or multiple rounded inclusions (see Fig. 9.2 and 42.5). They are observed in patients of G6PD deficiency after exposure to oxidizing drugs/chemicals.

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SUMMARY

- Reticulocytes are immature RBCs which are slightly larger than mature RBCs.
- Reticulocyte count may be performed either by visual method or more accurate automated method or by a flow cytometer.
- Supravital stains like brilliant cresyl blue/new methylene blue are used in visual methods which demonstrate blue filamentous or granular material in the reticulocytes.
- Reticulocyte count reflects the erythropoietic activity of the bone marrow.
- Supra vital stains are also used for demonstration of HbH inclusions and Heinz bodies.

SELF-ASSESSMENT EXERCISES

I. Short Note

Reticulocyte count

II. Multiple Choice Questions

1. **All of the following statements are true with regard to reticulocyte EXCEPT**
   A. It is performed by using supravital staining
   B. It can be performed by visual and automated method
   C. Normal reticulocyte count ranges from 0.5 to 1.5%
   D. Reticulocyte count is increased in aplastic anemia
2. **Reticulocytosis is NOT found in**
   A. Hereditary spherocytosis
   B. Paroxysmal nocturnal hemoglobinuria
   C. Anemia following acute bleeding
   D. Anemia of chronic renal failure

ANSWERS

1. D **2.** D

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CHAPTER 44 Hematocrit, Red Cell Indices and ESR Estimation

CHAPTER OUTLINE

- ❖ Hematocrit
- ❖ Red Cell Indices
- ❖ Erythrocyte Sedimentation Rate
- ❖ LE Cell Test

HEMATOCRIT

Introduction: Hematocrit **(packed cell volume)** is the ratio of the volume of red cells to that of the whole blood. It indicates relative volume of red cells and plasma (*e.g.* in anemia red cells are reduced with corresponding reduction in the hematocrit).

Methods of Estimation of PCV

- Macromethod using Wintrobe tube
- Micromethod using capillary tube
- Automated analyser

Wintrobe Method

Wintrobe tube (Fig. 44.1): It is a special thick walled glass tube measuring **11 cm in length** and an internal diameter of 2.5 mm, with a capacity of 1 ml. This is calibrated at 1 mm intervals up to 105-110 mm with bold markings from top as 0, 10, 20, 30,...100 for ESR and 100, 90, 80,...10, 0 for PCV. The markings are in descending order from the top for PCV estimation and markings in ascending order from the top for ESR determination.
Anticoagulants used: EDTA, dry heparin or double oxalate.

Principle

Anticoagulated whole blood is centrifuged at a standard speed (3500 RPM). RBCs which are heavier than white cells, platelets and plasma, sediment at the bottom and the volume of red cell mass denotes the hematocrit.

Method

- Collect the blood in an EDTA vial. Mix the blood sample properly so as to mix both plasma and cells.
- With the help of a Pasteur's pipette (dropper with long, thin capillary tube like nozzle) draw the blood from the vial.

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- Place the nozzle of pipette at the bottom of dry and clean Wintrobe tube.
- Slowly press the rubber teat of the pipette and fill the tube till the top mark of 100. Avoid any air bubble being trapped during filling of the tube.
- Centrifuge the tube at 3500 RPM for half an hour.
- Take the reading of packed red cells from bottom.
- It is expressed as percentage.

Different layers (Fig. 44.1)

- **Lower layer:** This consists of red blood cells (packed).
- **Middle layer:** It is also called buffy coat and is the thin red-gray layer between the red cells and plasma consisting of WBC and platelets.

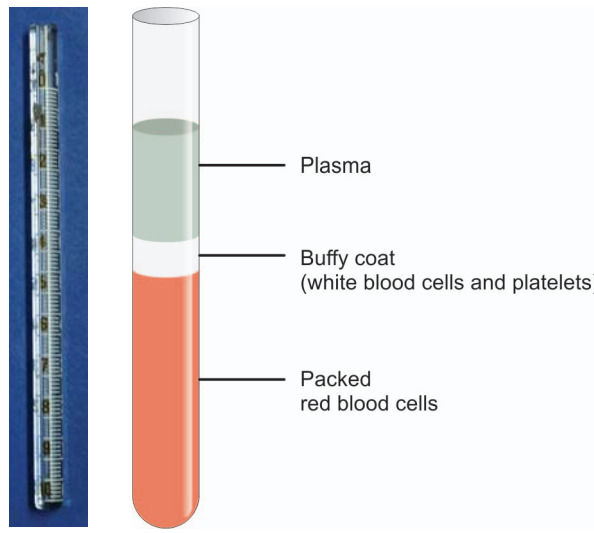


Fig. 44.1: Wintrobe tube (left) and different columns after the blood is centrifuged (right)

- **Upper layer:** This layer is composed of the plasma which is normally clear or straw colored.

Conditions causing changes in appearances of plasma are shown in Table 44.1.

Table 44.1: Interpretation of plasma column

Color	*Condition*
Clear/straw	Normal
Yellow (orange, green)	Jaundice
Opaque/turbid	Hyperlipidemia
Pink/red	Hemoglobinemia (hemolysis) as in PNH

Additional information from the Wintrobe method: PCV value is required to determine the red cell indices namely MCV and MCHC.

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Buffy coat gives an approximate indication of the number of WBCs (normally 0.1 mm of this layer = 1000 WBC/cu mm). Thickness of the buffy coat increases with marked leukocytosis, as in CML, CLL and acute leukemias. Absent or minimal buffy coat implies leukopenia.

Other uses of buffy coat

- Subleukemic leukemia—a smear made from the buffy coat has greater concentration of WBCs and identification of abnormal cells is easier.
- LE cell test

Note: Buffy coat is not useful for platelet estimation.

Other use of Wintrobe tube is for ESR estimation. However, the Westergren method is preferred as it is more accurate.

Microhematocrit Method

A heparinized capillary tube is used and the blood is filled by capillary action. One end is sealed and it is centrifuged in a special centrifuge. As the tubes are not graduated, the PCV is measured using a special chart.

Advantages

- Easy
- Small quantity of blood is needed (useful in pediatric patients).
- Capillary blood is sufficient.
- Less time
- Amount of plasma trapped in the red cell column is less, thereby minimizing the error.

Disadvantage

It requires a special (expensive) centrifuge.

Automated Analyser

Nowadays, automated analysers are available for estimation of hematocrit.

Normal range for PCV: Hematocrit is expressed as a percentage (e.g. 45%) or as a decimal fraction (e.g. 0.45). It is useful for evaluating absolute values like MCV and MCHC.

Males	40-50%
Females	36-46%
Infants	45-70% (cord blood)

Causes of Increased PCV (Table 18.1).

Decreased PCV is seen in anemia.

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Rule of Three

It is a rough guide to the accuracy of hemoglobin measurement as the **PCV should be about three times the Hb value**. When blood specimen are analyzed either by automated or manual method, a quick visual check of the results of the hemoglobin and hematocrit can be done by using the "rule of three". This rule applies only to normocytic normochromic RBCs. The value of hematocrit should be three times the value of the hemoglobin plus or minus 3.
Hemoglobin (gm/dL) × 3 = Packed cell volume (%) ± 3

RED CELL INDICES

Red cell indices are very useful in morphological characterization and diagnosis of anemias. The important red cell indices are

- Mean corpuscular volume (MCV)
- Mean corpuscular hemoglobin (MCH)
- Mean corpuscular hemoglobin concentration (MCHC)
- Red cell distribution width (RDW)

Mean Corpuscular (cell) Volume (MCV)

MCV is indicative of average volume of the red cell and is expressed in femtoliters or cubic micrometers

$$\text{MCV (fL)} = \frac{\text{PCV (\%)} \times 10}{\text{RBC count in millions}}$$

Normal range: 82-100 fL

Use: Classification and differential diagnosis of anemias.

Decreased MCV: Below 80 fL is seen in microcytic anemia.
Increased MCV: Above 100 fL is found in macrocytic anemia.

Note: In dimorphic anemias where both microcytic and macrocytic cells are present, MCV may be in the normal range. Similarly in cases with reticulocytosis, MCV is elevated resulting in false increase of MCV in microcytic anemias. Therefore, it is important to examine a peripheral blood smear.

Mean Corpuscular (cell) Hemoglobin (MCH)

MCH indicates the amount of Hb (weight) per red cell. It is expressed as picograms (1 pg = 10^{-12} g).

$$\text{MCH (pg)} = \frac{\text{Hb (in gm/dL)} \times 10}{\text{RBC count in millions}}$$

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Normal range: 27-32 pg
Use: It is of limited value in differential diagnosis of anemias.

Decreased MCH: Below 26 pg is seen in microcytic hypochromic anemias.
Increased MCH: Above 34 pg is found in macrocytic anemia, because the macrocytes are larger and carry more hemoglobin.

Mean Corpuscular (cell) Hemoglobin Concentration (MCHC)

MCHC denotes the average concentration of hemoglobin in the red cells. MCHC is actually a measurement of hemoglobin concentration in the red cell taking volume into account.

It is expressed as gm/dL (earlier it was expressed as %).

$$\text{MCHC (gm/dL)} = \frac{\text{Hb (in gm/dL)} \times 100}{\text{PCV (\%)}}$$

Normal range: 31-35 gm/dL
Use: It is a better indicator of hypochromasia than MCH.

Decreased MCHC: MCHC is less than 31 gm/dL in hypochromic red cells as observed in iron deficiency anemia and thalassemia syndromes.
Normal MCHC: It remains nearly normal in macrocytic anemia.
Increased MCHC: MCHC more than 35 gm/dL indicates hyperchromic red cells. Usually red cells do not contain more hemoglobin; however, microspherocytes because of their low volume do have relatively more hemoglobin and hence increased MCHC.

Red Cell Distribution Width (RDW)

Red cell distribution width (RDW) is an index of the variation in cell volume within the red cell population. It is one of the red cell indices and is provided by automated hematology analyser. It is the electronic quantitative measure of anisocytosis or variation in red blood cell size that is judged by peripheral smear examination. Mathematically, it is the coefficient of variation, i.e.

RDW = (Standard deviation ÷ mean cell volume) × 100

Normal range: 11.5-14.5%. A normal RDW indicates that red blood cells are relatively uniform in size. No subnormal values are found and an RDW below the reference interval is not a clinically relevant finding.

Uses

- A raised RDW indicates that the red cells are more variable in volume than normal. This may be due to the presence of smaller or larger red blood cells or a combination of these. For example, increased numbers of immature red blood cells during a regenerative response to

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an anemia will increase the RDW, because immature anucleate red blood cells are larger than normal.

- RDW in conjugation with MCV is used to subclassify the likely cause of anemia.
  - Most useful for distinguishing IDA from thalassemia. In early iron deficiency anemia, RDW increases along with low MCV while in thalassemia trait, RDW is normal with low MCV, thus distinguishing the two diseases from each other.
  - Characterization of dimorphic red cell populations (in vitamin B_{12}/folic acid and iron deficiency anemia), agglutination and fragmentation of red cells.

ERYTHROCYTE SEDIMENTATION RATE

Erythrocyte sedimentation rate (ESR) estimation is a commonly used nonspecific test in routine clinical practice.

Principle: When anticoagulated blood is placed in a vertical tube and is allowed to stand, RBCs settle towards the bottom of the tube. The speed of sedimentation of red cells in plasma over a period of 1 hour is measured by the length of the sedimented RBC column and is expressed in millimeters. RBCs have net negative charge on their surface and tend to repel each other. The repulsive forces are partially or totally counteracted if there is an increase in the positively charged plasma proteins.

Factors Affecting ESR

- **Plasma factors:** An accelerated ESR is favored by elevated levels of fibrinogen, globulins and cholesterol (which increase the positive charge of plasma) where as albumin and lecithin retard ESR.
- **Red cell factors:** The sedimentation rate is directly proportional to the weight of the cell aggregates and inversely proportional to the surface area.
  - **Number:** Anemia increases the ESR and polycythemia decreases.
  - **Size:** Microcytes sediment slower than macrocytes.
  - **Rouleaux formation:** It accelerates the ESR.
  - Red cells with an **abnormal or irregular shape**, such as sickle cells or spherocytes, do not exhibit rouleaux formation and have low ESR.
- **Technical factors:** ESR tube must be kept vertical, otherwise it results in inaccurate ESR.

Stages of ESR

Sedimentation occurs in three stages:

- **Stage of aggregation/rouleaux formation**: In the initial 10 minutes, there is little sedimentation as rouleaux form and the size of the rouleaux formed influence the speed of sedimentation.

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- **Stage of settling**: For about 40 minutes, settling occurs at a constant rate.
- **Stage of packing**: Packing of RBCs occurs in the final 10 minutes.

Methods

The two commonly employed methods are: Westergren and Wintrobe methods.

Westergren Method

Equipment (Fig. 44.2)

Westergren tube is a straight glass pipette (open at both ends) 30 cm in length, bore of 2.55 mm and calibrated in millimeters from 0-200. The capacity of tube is about 1 ml. The tube is vertically placed on the Westergren rack.

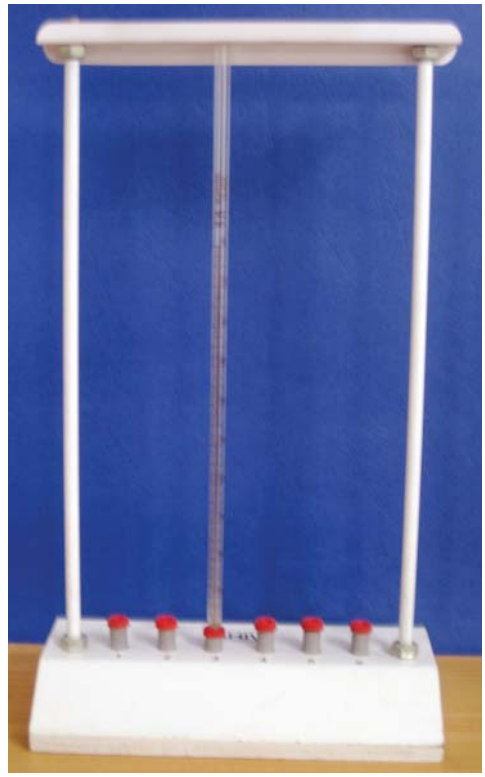


Fig. 44.2: Westergren pipette placed vertically in the rack

Anticoagulant used: 3.8% trisodium citrate

Ratio of blood and anticoagulant: 2 ml of whole blood in 0.5 ml of 3.8% trisodium citrate. Blood to anticoagulant ratio should be 4:1.

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Procedure

- A Westergren pipette is filled upto the 0 mark and placed vertically in the Westergren rack at room temperature.
- After 60 minutes, the distance between the top of the red cell column and 0 mark is noted in millimeters as ESR reading.

Result: ESR is expressed as mm at the end of one hour.

Normal Range (Table 44.2)

Table 44.2: Normal range for ESR

Adult males	0-10 mm	lst hour
Adult females	0-12 mm	lst hour
Children	0-10 mm	lst hour

Advantage

This is a more sensitive and accurate method when compared to the Wintrobe method. This is because the column is longer, i.e. 200 mm.

Disposable Methods

Disposable ESR kits are more convenient, where the ESR pipette sucks the blood to fill precisely to the zero mark. This eliminates risky mouth pipetting of blood.

Wintrobe Method

Wintrobe tube can be used first for estimation of ESR and then it is centrifuged for estimation of hematocrit.

Advantages

- It is simple and requires small amount of blood.
- Same tube can be used for both ESR and PCV.
- Buffy coat smear is useful in cases of leukopenia, subleukemic/aleukemic leukemia and for LE cell test.

Disadvantage

ESR value higher than PCV column can not be obtained by this method.

Alternative Methods

- Capillary method (micro ESR method) requires only 0.2 mL of blood, so it is useful in pediatric patients. The results are precise and reading time is 20 minutes.
- Zeta Sedimentation Rate
- Automated ESR (e.g. Ves-Matic benchtop analyser): Results are obtained within 30 minutes and are comparable with Westergren 1 hour reading.

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Significance of ESR

ESR is a prognostic test rather than a diagnostic test. It is useful in monitoring disease activity. Note:

- ESR may be rarely normal in patients with neoplasms, connective tissue diseases and infections. A normal ESR cannot be used to exclude these diagnostic possibilities.
- ESR is one of the Jones minor criteria for the diagnosis of rheumatic fever.

Causes of Increased ESR (Table 44.3)

Table 44.3: Causes of increased ESR

Markedly increased in:
- Multiple myeloma
- Macroglobulinemia
- Hyperfibrinogenemia

Moderately increased in:
- Infective diseases like tuberculosis
- Chronic inflammatory diseases
  - Rheumatic fever
  - Autoimmune diseases: Rheumatoid arthritis,SLE, etc.
- Neoplasia

Causes of Decreased ESR

- Polycythemia vera
- Sickle cell disease
- Hypofibrinogenemia

LE CELL TEST

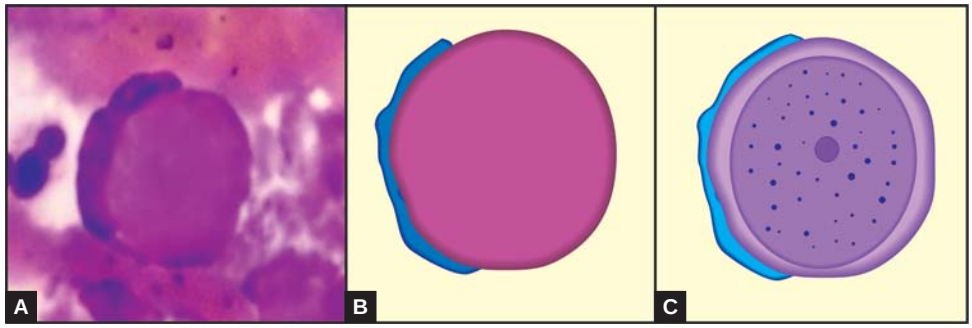
In autoimmune diseases (e.g. SLE, rheumatoid arthritis), variety of antibodies are found. One of them is an antinuclear antibody (ANA) which cannot penetrate intact cells. ANAs can react with nuclei of damaged cells and converts nuclear chromatin into homogeneous material. *In vivo,* ANAs act on the nuclei of damaged cells and denatures it into homogeneous material. This nuclear material is phagocytosed in the presence of complement by phagocytic leukocyte (neutrophil or macrophage). The LE cell (Figs 44.3A and B) is any phagocytic leukocyte (neutrophil or macrophage) that has engulfed the denatured nucleus of an injured cell. In the tissue they are known as LE bodies or hematoxylin bodies.

Tart cell: LE cell has to be differentiated from a tart cell (Fig. 44.3C). It is a monocyte/neutrophil containing a phagocytosed nucleus of another cell. The nuclear material is not homogeneous and it retains its chromatin pattern. It may be found in healthy individuals.

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Significance: LE cells may be demonstrated in SLE, rheumatoid arthritis, other autoimmune disorders, hepatitis and penicillin sensitivity.



Figs 44.3A to C: LE cell (A and B) and tart cell (C)

SUMMARY

- PCV is a measure of packed red cell volume and is one of the investigations helpful in hematology.
- Red blood cell indices include the MCV, MCH, MCHC and RDW. They are helpful in determining the etiology of anemia.
- ESR is a nonspecific test which measures the rate of settling of red cells in 1 hour. It is mainly useful as a prognostic test to monitor the therapy in certain diseases.
- LE cell is phagocytic leukocyte that has engulfed the denatured nuclear material of an injured cell and is found in autoimmune diseases like SLE and rheumatoid arthritis.

SELF-ASSESSMENT EXERCISES

I. Short Notes

1. Packed cell volume/estimation of PCV
2. Uses of buffy coat
3. PCV tube and its uses
4. Buffy coat
5. Red cell indices
6. Erythrocyte sedimentation rate and interpretation
7. Westergren pipette
8. Stages of ESR
9. Anticoagulants used for ESR
10. LE cell

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II. Multiple Choice Questions

1. **PCV can be estimated by the following methods EXCEPT**
   A. Macromethod using Wintrobe tube
   B. Micromethod using capillary tube
   C. Westergren tube
   D. Automated analyser
2. **Buffy coat is useful for demonstration of**
   A. LE cells
   B. Antiplatelet antibodies
   C. Erythrophagocytosis
   D. Toxic granulation
3. **Micromethod of PCV estimation is useful in**
   A. Pediatric patients
   B. Comatose patients
   C. Patients in intensive care unit
   D. Aged patients
4. **Buffy coat is useful**
   A. In LE cell preparation
   B. For demonstration of hypersegmented neutrophils
   C. For detecting malarial parasite
   D. For detecting microfilaria
5. **RDW in conjunction with MCV is useful in distinguishing**
   A. Iron deficiency anemia from thalassemia
   B. Iron deficiency anemia from sickle cell anemia
   C. Sickle cell anemia from thalassemia
   D. Thalassemia major from minor
6. **Raised ESR is found in**
   A. Sickle cell anemia
   B. Multiple myeloma
   C. Polycythemia vera
   D. Congestive heart failure
7. **Blood to anticoagulant ratio in ESR estimation by Westergren method is**
   A. 1:5
   B. 5:1
   C. 4:1
   D. 1:4
8. **Anticoagulant used for ESR estimation by Westergren method is**
   A. Double oxalate
   B. Heparin
   C. Trisodium citrate
   D. Fluoride

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9. Anticoagulant used for ESR estimation by Wintrobe's method is
A. Heparin
B. EDTA
C. Fluoride
D. Trisodium citrate

10. ESR is reduced in
A. Rheumatic fever
B. Sickle cell anemia
C. Rheumatoid arthritis
D. Active tuberculosis

ANSWERS

1. C **2.** A **3.** A **4.** A **5.** A **6.** B **7.** C **8.** C
9. B **10.** B

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CHAPTER 45 Bone Marrow Examination

CHAPTER OUTLINE

- Introduction
- Bone Marrow Aspiration
- Bone Marrow Trephine Biopsy

INTRODUCTION

Bone marrow examination is essentially done to confirm or rule out a hematologic disorder. It also helps in evaluation of non-hematological disorders (e.g. metastasis).

Bone marrow may be obtained by:

- **Aspiration:** Bone marrow aspiration is a simple, easy and safe procedure.
- **Trephine biopsy** is indicated in conditions where the aspiration either fails to yield marrow or to confirm some of the diseases (where biopsy findings are diagnostic).

BONE MARROW ASPIRATION

Bone Marrow Needles

Needles commonly employed for the aspirations of the marrow are Salah needle (Fig. 45.1) and Klima needle.

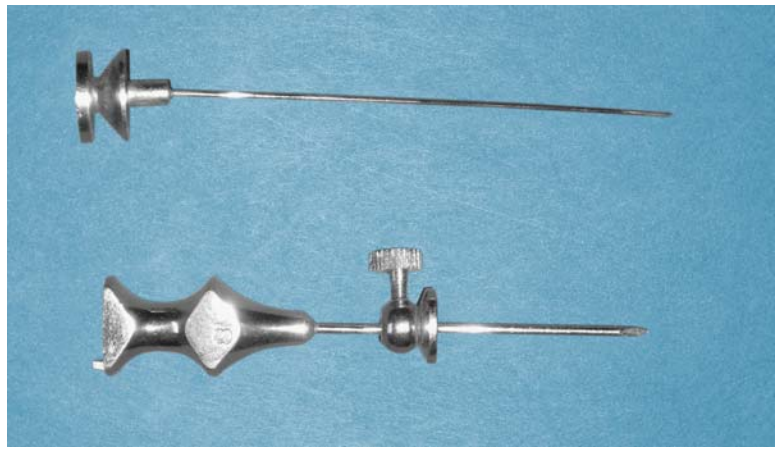


Fig. 45.1: Salah bone marrow aspiration needle with stilette (above)

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Parts of the Bone Marrow Needle

- ***Stilette:*** It prevents entry of soft tissue and bone fragments into the aspiration needle when it pierces through them. The stilette has to be removed when the needle reaches the marrow space.
- ***Aspiration needle:*** For aspirating the marrow, a syringe is attached after removing the stilette.
- ***Guard:*** It adjusts the depth of penetration of the aspiration needle.

Sites for Bone Marrow Aspirate

Usual sites for bone marrow aspiration are
- Sternum
- Posterior superior iliac spine
- Iliac crest
- Anterior superior iliac spine
- Spinous process of lumbar vertebra.

In **infants**, upper end of the tibia is the ideal site for marrow aspirate.

Evaluation of Bone Marrow Aspirate

- **Cellularity:** The cellularity of the marrow is reported as normocellular/hypercellular/hypocellular based on the microscopic examination of marrow particle.
  Cellularity of marrow should be correlated with the age of the patient.
- **M**yeloid and **E**rythroid **(M:E) ratio**
  This is done by counting at least 500 cells and expressing the cells as ratio of myeloid and erythroid cells. It provides quantitative information about the relative proportion of erythroid and myeloid precursors. Normal M: E ratio varies from 2:1 to 4:1.
- **Erythropoiesis:** Erythroid precursors are examined for the
  - Marrow response: Normal/increased/decreased
  - Maturation: Normoblastic/megaloblastic/micronormoblastic
  - Dyserythropoiesis
- **Myelopoiesis:** It is examined for
  - Normal response: Normal/increased/decreased
  - Maturation arrest
  - Morphological abnormalities: Hypergranular/absent granules
  - Abnormal cells/blasts
- **Megakaryopoiesis:** They are examined for
  - Number
  - Morphology
- **Lymphocytes:** Normal/increased/abnormal
- **Plasma cells:** Normal/increased

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- **Others:**
  Metastatic tumor cells, parasites (malaria, Leishmania donovani) and fungus or any other abnormal cells.
- **Iron Stores**
  Iron stores are evaluated by Prussian blue reaction (Perl's stain).
  Iron stores are commented as normal/increased/decreased or nil.
- **Cytochemistry/special stains.**

Indications for Bone Marrow Aspiration

- *Diagnostic*
  - **Primary hematolymphoid disorders**
    - **Red cell disorders:** Nutritional anemia (e.g. megaloblastic anemia), pure red cell aplasia
    - **White cell disorders:** Subleukemic/ aleukemic leukemia, diagnosis and classification of acute leukemias
    - **Megakaryocytic disorders:** ITP and other thrombocytopenias
    - **Myeloproliferative neoplasms:** Polycythemia vera, chronic myeloid leukemia, idiopathic thrombocythemia
    - **Myelodysplastic syndromes**
    - **Plasma cell neoplasms:** Multiple myeloma, Waldenström macroglobulinemia
  - **Systemic diseases**
    - **Storage disorders:** Gaucher, Niemann-Pick disease
  - **Staging of lymphoid malignancies and solid tumors**
    - Lymphoma
    - Metastatic deposits (e.g. carcinoma prostate, breast, lung, kidney)
  - **Detection of infection and/or source of PUO**
    - **Parasitic disorders:** Kala-azar
    - **Fungal disorders:** Histoplasma
    - **Mycobacterial infection**
  - **Iron stores**
  - **Miscellaneous disorders:** Pancytopenia or unexplained cytopenias
- ***Post-treatment follow up:*** To know the response to therapy and follow up in cases of leukemia, aplastic anemia and agranulocytosis.
- ***Therapeutic:*** Bone marrow transplant

Contraindications

- Hemophilia
- Congenital hemorrhagic disorders.

Dry Tap

During aspiration, if the marrow is not obtained it is called a dry tap. Dry tap is common in hairy cell leukemia and myelofibrosis (marrow has been replaced by fibrous tissue).

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BONE MARROW TREPHINE BIOPSY

Marrow trephine biopsy is performed by one of the trephine biopsy needles like Jamshidi needle or Westerman-Jensen needle or Islam needle. The biopsy obtained consists of a core of bone with marrow. Biopsy material is rolled between two glass slides and an imprint smear is immediately prepared. These are excellent for morphological evaluation as well as for special stains. Trephine biopsy tissue is decalcified and processed like other histopathological tissue containing bone. Sections are stained with hematoxylin and eosin stain, reticulin stain, Masson's trichrome stain (for fibrous tissue). Immunocytochemical staining can be performed especially for acute leukemias.

Sites of Trephine Biopsy

- Posterior superior iliac spine (most commonly used site)
- Anterior superior iliac spine
- Spinous process of vertebra.

Indications for Trephine Biopsy

- Aplastic anemia
- Myeloproliferative neoplasms- to study reticulin fibrosis in myelofibrosis.
- Myelodysplastic syndromes
- Pre- and post-bone marrow transplantation
- Pyrexia of unknown origin (granuloma of tuberculosis)
- Bone morphology in chronic renal failure, osteoporosis and osteomalacia.
- Staging
  - Lymphoma
  - To detect metastasis in cancer patients.
- AIDS.

SUMMARY

- Bone marrow examination is a valuable adjunct to peripheral smear examination in the evaluation of both hematologic and non-hematological disorders.
- Marrow may be obtained either by aspiration or by trephine biopsy.
- Marrow aspiration can be done for diagnostic, follow up or therapeutic purpose.
- Marrow should be evaluated in a systematic way.
- Examine for cellularity, erythropoiesis, myelopoiesis, M:E ratio and megakaryopoiesis.
- Also look for any abnormal cells and iron stores.
- Trephine biopsy may be required in certain diseases like aplastic anemia, myeloproliferative neoplasm, staging of lymphoma, etc.

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SELF-ASSESSMENT EXERCISES

I. Short Notes

1. Indications for bone marrow aspiration
2. Absolute indications for bone marrow aspiration
3. Indications for bone marrow trephine biopsy
4. Needles used for bone marrow biopsy

II. Multiple Choice Questions

1. **Ideal site for bone marrow aspirate in infants is**
   A. Sternum
   B. Spinous process of lumbar vertebra
   C. Upper end of the tibia
   D. Lower end of femur
2. **Ideal site for bone marrow aspirate in adults is**
   A. Sternum
   B. Spinous process of lumbar vertebra
   C. Posterior superior iliac spine
   D. Upper end of the tibia
3. **Bleeding disorder in which bone marrow aspiration done for diagnosis is**
   A. Thrombocytopenia
   B. Hemophilia
   C. Henoch-Schönlein purpura
   D. von Willebrand disease
4. **The following disease is a contraindication for bone marrow aspiration**
   A. Immune thrombocytopenic purpura
   B. Hemophilia
   C. Bone marrow metastasis
   D. Henoch-Schönlein purpura
5. **Dry tap during bone marrow aspiration is most likely observed in**
   A. Acute lymphoblastic leukemia
   B. Multiple myeloma
   C. Pernicious anemia
   D. Myelofibrosis
6. **Bone marrow trephine biopsy is advantageous than aspiration for**
   A. Assessing the architecture of the marrow
   B. Assessing the morphological features of marrow cells
   C. Performing special stains
   D. Assessing the bone marrow iron

ANSWERS

1. C **2.** C **3.** A **4.** B **5.** D **6.** A

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CHAPTER 46

Osmotic Fragility Test

CHAPTER OUTLINE

- Introduction
- Principle
- Procedure
- Interpretation

INTRODUCTION

The normal red cell membrane is unstretchable and is freely permeable to water. RBCs are not lysed in normal buffered (pH 7) saline solution (9 gm/L NaCl). The osmotic fragility test measures the ability of RBCs to withstand lysis when suspended in buffered solutions of various concentrations of sodium chloride (NaCl). The pattern of lysis helps to determine whether the shape of RBCs is normal or abnormal. Spherocytes and stomatocytes hemolyze with higher concentration of NaCl compared to normal red blood cells.

PRINCIPLE

- In this test, small volumes of blood are mixed with large excess of buffered saline of varying concentration.
- When the red cells are suspended in buffered hypotonic solutions (i.e. the strength of the saline solution is decreased below normal saline), their volume progressively increases till a "critical hemolytic volume" is reached. At this critical point, the red cell membrane ruptures and liberates hemoglobin into the supernatant solution. The ability of normal RBCs to withstand hypotonic solution, lies in its biconcave shape which allows considerable water to enter the RBC before it stretches enough to break or lyse.
- If RBCs are spherocytic, there is loss of membrane which causes reduction in their surface area. The spherocytes can not expand as much as normal biconcave RBCs and there is decrease in the critical hemolytic volume. When the strength of the saline solution is decreased (hypotonic) spherocytic red cells (which do not have normal biconcave shape) will begin to **hemolyze more than normal red cells.** The sooner hemolysis occurs, the greater the osmotic fragility of the cells. Incubation of blood overnight at 37°C makes the osmotic fragility test (OFT) more sensitive.

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PROCEDURE

- A series of test tubes containing solutions of NaCl with varying concentration ranging from 0.9-0.10% (0.9, 0.75, 0.65, 0.6, 0.55, 0.5, 0.4, 0.35, 0.3, 0.2 and 0.10%) are taken.
- Red cells are suspended in each tube and incubated at room temperature for 30 minutes, and then centrifuged.
- The percentage of hemolysis in the supernatant solutions of each tube (with various concentrations) is assessed photoelectric colorimeter. The results are plotted in a graph, placing % of NaCl solution on horizontal axis and % of lysis on the vertical axis (Fig. 46.1).
- The reading of the supernatant from tube 0.9% is taken as blank and the reading of supernatant of tube 0.1% saline as 100%.

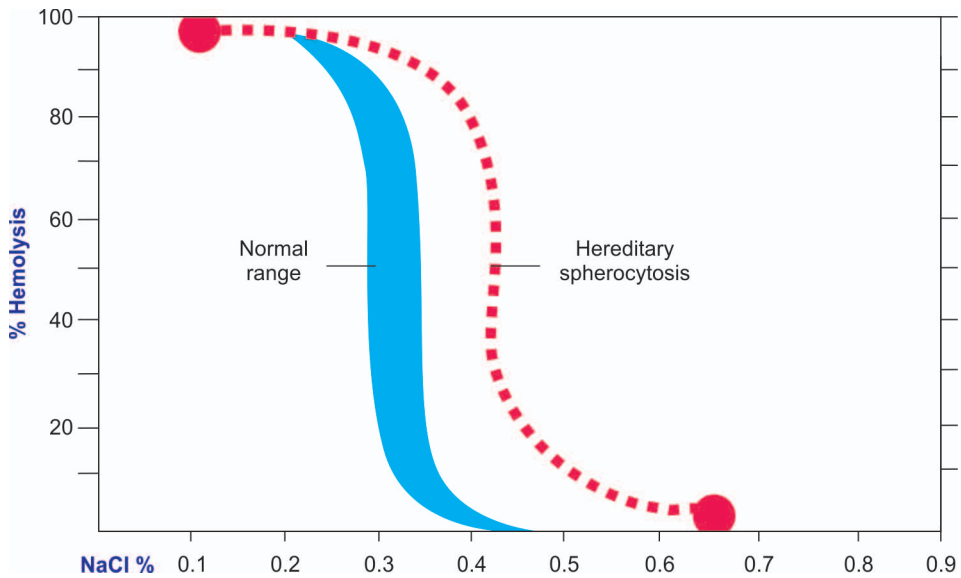


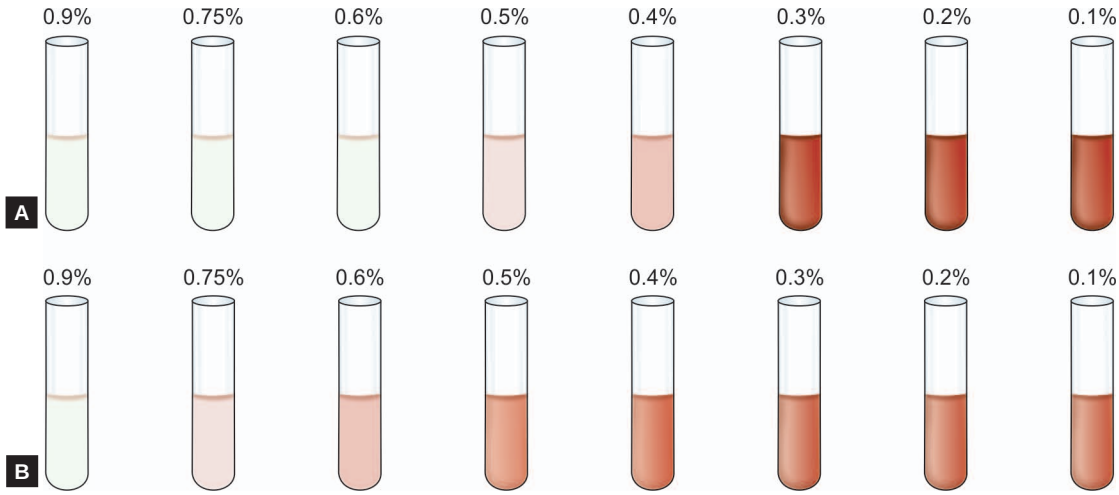
Fig. 46.1: Osmotic fragility test. Normal curve (blue) and increased osmotic fragility in hereditary spherocytosis

INTERPRETATION

- **Normal:** Lysis of fresh RBCs begins at 0.5% of NaCl solution and is complete at 0.3% (Fig. 46.2A).
- **Increased osmotic fragility:** Spherocytes having decreased surface/volume ratio, have a limited capacity to expand in hypotonic solutions. Lysis of spherocytes begins early at a higher concentration of NaCl (shown at 0.75% in Fig. 46.1 and 46.2B) than do normal biconcave red cells. This is known as **increased osmotic fragility** and shows a **shift to right** of normal range in the graph (Fig. 46.1). Increased osmotic fragility indicates that the red cells are spherocytic. Increased osmotic fragility is also observed in hereditary spherocytosis, hereditary elliptocytosis and other conditions associated with spherocytosis, like in autoimmune hemolytic anemia.

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Figs 46.2A and B: (A) Normal osmotic fragility of fresh RBCs in which hemolysis begins at 0.5% and completes at 0.3%. (B) Abnormal hemolysis beginning even at mild dilution (0.75%) in hereditary spherocytosis. (Note: Only few different concentrations of NaCl solutions are shown)

- **Decreased osmotic fragility:** Conversely, hypochromic and flat red cells have an increased capacity to expand in hypotonic solutions. They lyse at a lower concentration than do normal cells, and thus have decreased **osmotic fragility**. The graph shows **shift to the left** of normal range and indicate that the red cells are flattened or hypochromic. This type of RBCs have increased surface/volume ratio and are found in iron deficiency, thalassemia, sickle cell anemia, liver disease and reticulocytosis.

Use

For the diagnosis of of hereditary spherocytosis.

SUMMARY

- Osmotic fragility test measures the lysis of RBCs suspended in buffered solutions of various concentrations of NaCl.
- Increased osmotic fragility is found in spherocytosis and stomatocytosis which hemolyze with higher NaCl concentration than normal red blood cells.

SELF-ASSESSMENT EXERCISES

I. Short Note

Osmotic fragility test and its importance

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II. Multiple Choice Question

1. **Osmotic fragility is increased in**
   A. Pernicious anemia
   B. Iron deficiency anemia
   C. Hereditary spherocytosis
   D. Chronic lead poisoning

ANSWER

1. C

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Chapter 47 Cytochemistry in Leukemia

Chapter Outline

- Definition
- Myeloperoxidase
- Sudan Black B
- Nonspecific Esterase
- Periodic Acid Schiff Reaction
- Neutrophil Alkaline Phosphatase

DEFINITION

Cytochemistry is the study of chemical elements found in the cytoplasm of the cells. The chemical element in the cell may be an enzyme (e.g. myeloperoxidase) or nonenzymatic substance (e.g. lipids and glycogen).

Main Uses of Cytochemistry

- To characterize the blast cells as myeloid or lymphoid in acute leukemias. Sometimes, differentiating AML from ALL is not possible only on morphological grounds and in such cases cytochemical stains will be of help.
- To identify granulocytic and monocytic components of acute myeloid leukemia
- To detect cytoplasmic abnormalities and enzyme deficiencies in myeloid disorders.

The various cytochemical stains used are:

MYELOPEROXIDASE

Myeloperoxidase (MPO) is an enzyme present in the primary granules of **mature and immature** cells of **myeloid series.**

Principle: When hydrogen peroxide is present, the myeloperoxidase within the cytoplasm oxidizes the dye substrate creating brown granules in the cytoplasm (see Fig. 23.2A) and the intensity of positivity increases with maturity of the cell.

Interpretation

- Leukemic myeloblasts are positive. (AML M_1, M_2, M_3 and M_4 blasts and Auer rods).
- Lymphoblasts and normoblasts are negative. Thus, useful in differentiating AML from ALL.

Precaution: Reaction is to differentiate only blast cells and not for maturing granulocytes which are normally positive.

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SUDAN BLACK B

Principle: Sudan black B (SBB) stains lipids and phospholipids present in the granules of the cells of **myeloid series.** The granules take up black color (see Fig. 23.2B). Staining pattern in MPO and SBB are similar.

Interpretation

- Useful in differentiation of AML from ALL.

NONSPECIFIC ESTERASE

Esterase is used to differentiate **myeloblast, neutrophilic precursors and neutrophilic granulocytes** from monocytic cells.

Principle: Esterases are enzymes in cells of monocytic series and are used to differentiate them from myeloblasts and lymphoblasts.

Interpretation

- Esterase is useful for identification and confirmation of the monocytic component in AML M_4 and M_5. Monocytic cells show dark red cytoplasmic granules.

PERIODIC ACID SCHIFF REACTION

Periodic acid Schiff (PAS) stain is useful for the diagnosis of ALLs, and erythroid and megakaryocytic (M_6 and M_7) type of acute myeloid leukemia.

Principle: Many cells contain glycogen in their cytoplasm. The periodic acid oxidizes glycogen, mucoproteins and other high molecular weight (HMW) carbohydrates to aldehydes. These aldehydes react with colorless Schiff reagent and take bright-red pink (*magenta)* color. The staining pattern may be fine and diffuse, coarse and granular (block positivity), or a mixture of both patterns.

Interpretation: Useful for differentiating AML from ALL.

- Myeloblasts are negative. Erythroid type of acute myeloid leukemia may show PAS—positive erythroblasts.
- **Lymphoblasts show blocks of PAS positive** material in the cytoplasm (see Fig. 22.1A inset). Block positivity in lymphoblasts is observed in most of the ALL L_1 cases.

Cytochemical stains useful in typing the blast cells are presented in Table 47.1.

Table 47.1: Cytochemical stains useful in typing the blast cells

Stain	*Precursor B-ALL*	*Precursor T-ALL*	***AML***		
			M_1-M_3	*M_4-M_5*	*M_6-M_7*
Myeloperoxidase	Negative	Negative	+/++	+	Negative
Sudan Black	Negative	Negative	+/++	+	Negative
PAS	+(coarse)	Negative	Negative	+	+(fine)
Nonspecific esterase	Negative	Negative	Negative	++	+(focal)
Acid phosphatase	Negative	+(focal)	Negative	+ diffuse	+(focal)

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NEUTROPHIL ALKALINE PHOSPHATASE

Principle: Alkaline phosphatase is an enzyme located in the specific (secondary) or tertiary granules present in the cytoplasm of mature neutrophils, with some activity in metamyelocytes. The positive reaction gives rise to blue and granular appearance to the cytoplasmic granules. (Fig. 47.1).

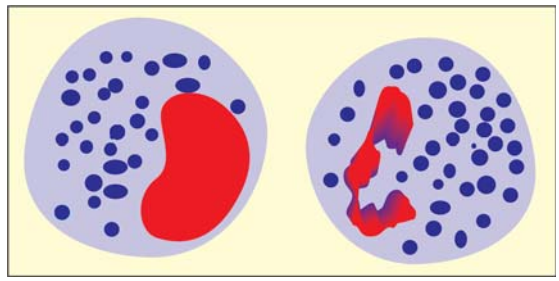


Fig. 47.1: Metamyelocyte (left) and neutrophil (right) show positive reaction (blue and granular) with neutrophil alkaline phosphatase

NAP/LAP Score

Normal NAP/LAP score: 40-100

Increased

- Hematological disorders
  - Leukemoid reaction
  - Myelofibrosis
  - Polycythemia rubra vera
  - Idiopathic thrombocythemia.
- Nonhematological disorders
  - Pregnancy
  - Down syndrome.

Decreased

- Paroxysmal nocturnal hemoglobinuria
- Chronic myeloid leukemia.

SUMMARY

- Cytochemistry is the study of chemical elements found in the cytoplasm of the cells. The chemical element in the cell, may be enzyme or nonenzymatic substance.
- Whenever differentiating AML from ALL is not possible on morphological grounds, cytochemical stains will be of help.
- Myeloperoxidase is an enzyme present in the primary granules of mature and immature cells of myeloid series. Leukemic myeloblasts are positive and lymphoblasts are negative.

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- Sudan black B stains lipids and phospholipids present in the granules of the cells of myeloid series.
- Nonspecific esterase is used to differentiate myeloblast and neutrophilic granulocytes from monocytic cells.
- Periodic Acid Schiff (PAS) reaction is negative in myeloblasts. Lymphoblasts show blocks of PAS positive material in the cytoplasm.
- Neutrophil alkaline phosphatase is raised in leukemoid reaction and helps in differentiating it from chronic myelogenous leukemia, in which it is decreased.

SELF-ASSESSMENT EXERCISES

I. Short Note

Use of cytochemistry in leukemia

II. Multiple Choice Questions

1. **Blasts which show block positivity with PAS, stains are**
   A. Myeloblasts
   B. Monoblasts
   C. Megaloblasts
   D. Lymphoblasts
2. **The hematological disorder NOT associated with increased LAP score is**
   A. Leukemoid reaction
   B. Myelofibrosis
   C. Polycythemia rubra vera
   D. Chronic myeloid leukemia
3. **Leukocyte alkaline phosphatase is increased in**
   A. Acute myeloid leukemia
   B. Chronic myeloid leukemia
   C. Myeloid metaplasia
   D. Myeloid leukemoid reaction

ANSWERS

1. D **2.** D **3.** D

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CHAPTER 48
Laboratory Evaluation of Hemostatic and Thrombotic Disorders

CHAPTER OUTLINE

- Introduction
- Tests for Platelet Component
- Tests for Platelet and Vascular Component
- Tests for Coagulation Component
- Tests for Fibrinolytic Activity

INTRODUCTION

Disorders of hemostasis are broadly divided into bleeding disorders and thrombotic disorders (see Table 34.1). The clinical laboratory evaluation is an integral part of the diagnosis and management of patients with bleeding/thrombotic disorders. Laboratory tests for hemostatic/thrombotic disorders depend on the component of hemostasis that is involved (Table 48.1).

Table 48.1: Laboratory tests for hemostatic/thrombotic disorders depending on the component involved

Tests for bleeding disorders			***Tests for thrombotic disorders***	
Platelet component	*Plate-vessel wall interaction*	*Coagulation component*	*Coagulation regulatory system*	*Fibrinolytic activity*
Platelet count Platelet aggregation test Clot retraction test	Capillary fragility test (tourniquet test) Bleeding time	Clotting time Prothrombin time (PT) Activated partial thromboplastin time (APTT) Thrombin time (TT) Prothrombin consumption time Factor assay	Antithrombin Proteins C and S Tissue factor pathway inhibitor (TFPI)	Whole blood clot lysis Euglobulin lysis time Fibrinogen/fibrin degradation products D-dimer

Most bleeding disorders are caused by one of three defects: (1) a defect in platelet number or function, (2) a defect in platelet -vessel wall interactions (i.e. an abnormality in the adhesive interactions between platelets and the vessel wall) or (3) a defect or deficiency in coagulation factor. The diagnosis of bleeding disorders requires a battery of tests (Table 48.2) which include screening tests done in all patients with history of bleeding, followed by specific tests to identify the exact nature of the disorder.

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Table 48.2: Laboratory tests in bleeding disorders

Screening tests	*Accessory tests*	*Others*
Platelet count Bleeding time Prothrombin time (PT) Activated partial thromboplastin time (APTT) Thrombin time (TT)	Capillary fragility test (tourniquet test) Clotting time Clot retraction test	Prothrombin consumption time Factor assay

TESTS FOR PLATELET COMPONENT

Platelet Count

It is obtained on anticoagulated blood either manually or by electronic particle counters (Chapter 41).

Normal range of platelet count: 1,50,000 to 4,50,000 platelets/cu mm.

Precaution: When the platelet counts are beyond the normal range, they should always be verified by examining the peripheral smear. This is because clumping of platelets can result in spurious low count during automated counting. Increased platelet count is usually seen in myeloproliferative neoplasms (e.g. essential thrombocythemia).

Causes of thrombocytopenia and thrombocytosis are discussed in Chapters 35 and 41.

Platelet Aggregation

Principle: These tests measure the ability of platelets to aggregate in response to agonists like thrombin and form the basis for qualitative tests of von Willebrand factor.

***Use*:** For classification of congenital qualitative disorders of platelets.

Clot Retraction Test

Principle: After the coagulation of blood, the clot under the action of thrombasthenin (a substance released from platelets) undergoes contraction and starts retracting within one hour. The clot shows 50% retraction in 2 to 4 hours. This process is completed in 18 to 24 hours with separation of serum. Subsequently, the fibrin clot dissolves due to fibrinolysis and the RBCs sink to the bottom. Clot retraction is dependent on normal platelet number, platelet function, concentration of fibrinogen and the activity of the fibrinolytic pathway.

Procedure

- Place a tube containing blood in a water-bath at 37°C.
- Examine the clot after 1 hour and after 24 hours.

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- Note the size, consistency of the clot and the nature of retraction (normal or reduced).
- Continue observation of clot for 72 hours to assess the clot lysis.

Normal value: Normal clot retraction shows more than 50% of serum separated at the end of 24 hours. A normal clot is firm, rubbery, elastic and not easily broken.

Interpretation: Absent or reduced clot retraction is seen in:
- Fibrinogen deficiency (congenital or acquired)
- Thrombocytopenia
- Thrombasthenia.

TESTS FOR PLATELET AND VASCULAR COMPONENT

Capillary Fragility Test (Hess test/tourniquet test)

Principle: It measures the ability of capillaries to withstand the increased stress.

Procedure

- Sphygmomanometer cuff is tied to the upper arm above the elbow and the cuff is inflated to 80 mm for 5 minutes.
- Release the pressure after 5 minutes.
- The number of petechiae present in a circle of 5 cm diameter on the flexor aspect of forearm (below the bend of the elbow) is noted.

Normal: 0 to 5 petechiae.

Interpretation: Positive test is indicated by more than 10 petechiae and is found in:
- Vessel wall abnormalities:
  - Vascular purpura
  - Scurvy
- Platelet disorders:
  - Thrombocytopenia
  - Defective platelet function.

Bleeding Time (BT)

Bleeding time is used as screening test for disorders of platelet-vessel wall interactions. It measures the time required for bleeding to stop after a standardized superficial cut of the skin capillary bed.

Methods

- Duke's method-obsolete
- Ivy's method
- Template method (method of choice).

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Template Method for BT

Template is a disposable blade fitted on to a holder made of plastic and is used for the test. The blade projects through the bottom so that the incision made through the slit is 9 mm long and 1 mm deep.

Principle: A small skin cut of a standard size and depth is made and the oozing blood is wiped with a filter paper. Bleeding stops when the capillaries contract and platelet plug seals the vessel.

Procedure

- Patient is made to sit on a chair with an arm rest, so that the forearm is steady and exposed.
- The skin of the forearm is cleaned with alcohol and allowed to dry.
- Sphygmomanometer cuff is tied to the upper arm and the cuff is inflated to 40 mm Hg.
- A site on the forearm is selected away from the superficial veins.
- Place the template 5 cm distal to antecubital crease (on volar surface).
- One cut is made using a smooth rapid movement with the template (in the *simplate/surgicutt* test, no pressure is applied to the device. On pressing the trigger an incision of 5 mm length and 1 mm depth develops).
- Immediately after the incision, a stop watch is started.
- Another cut is made 1.5-2 cm away from the first one and another stop watch is started.
- The blood oozing from the sides of the cut is blotted with filter paper (Whatman No.1), every 30 seconds until bleeding stops. Do not touch the wound edges to avoid disturbing the clot as it is formed.
- Time at which bleeding stops is noted for both cuts.
- Place a butterfly adhesive bandage over the site of puncture to avoid scarring.
- Average of the two readings is the bleeding time for the patient.

Normal Range: 2-9 Minutes

Uses

- The test is prone to problems of reproducibility, sensitivity, and specificity. Though it is one of the screening tests, it is not recommended as routine preoperative screening test.
- This test evaluates the defects of primary hemostasis. Thus, bleeding time measures the platelet-vessel wall interactions (integrity of capillary and platelet function).

  Note: The bleeding time usually is not prolonged in patients with coagulation factor deficiencies.

Interpretation

Prolonged bleeding time is found in:

- **Platelet disorders**
  - **Quantitative:** Thrombocytopenia. If platelet count is below 50,000/ mm^3, BT should not be performed as bleeding may be difficult to stop.
  - **Qualitative:** von Willebrand disease, Bernard-Soulier syndrome, Glanzmann's thrombasthenia.

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- **Primary vascular disorders:** Ehlers- Danlos syndrome.
- **Platelet-vessel wall interactions:** von Willebrand disease
- **Others:** Afibrinogenemia, severe hypofibrinogenemia, uremia, aspirin.

TESTS FOR COAGULATION COMPONENT

Coagulation or Clotting Time (Lee-White Method)

It measures the time taken for the fresh blood to clot.

Procedure

- Draw 3 mL of venous blood with aseptic precautions.
- Label 3 test tubes as No. 1, 2 and 3 and keep in a water bath at 37°C.
- Deliver 1 mL of blood into each of the above 3 test tubes and start the stop watch.
- After 3 minutes, take out tube No 1, tilt it every 30 seconds till a clot develops.
- Note the time when the tube can be inverted completely.
- Next examine the tube No 2 every 30 seconds, exactly the same way as tube No 1, till the clot forms and note the time.
- Finally, invert the third test tube as above till blood clots. Stop the watch.
- Record the time from the moment blood is delivered into the test tube to the complete clotting in the third tube.
- The clotting time of the third tube is reported as the clotting time.

Normal: 4-11 Minutes

Disadvantages

Not a sensitive test: It is one of the oldest tests and is not sensitive as it fails to detect mild/moderate procoagulant defects. Hence, it is obsolete now and not recommended as a screening test. PTT is a more sensitive test for assessment of the coagulation cascade. Clotting time is prolonged only with severe deficiency of factor VIII, IX or fibrinogen (afibrinogenemia) and in heparin therapy.

Misleading: Normal value may be obtained in mild-to-moderately severe hemophilia A and B and it does not exclude major factor deficiency.

- **Other use:** Following the clotting time, the tubes can be left in the water-bath and examined after one hour for *clot retraction test.*

Specimen Collection for Coagulation Studies

- Care should be taken not to hemolyse the sample either by excessive suction during venipuncture or while delivering the blood through the needle into the vial.

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- Ratio between blood and anticoagulant should be exactly 1:9. Any variability of ratio affects the results of coagulation studies. Vacuum tubes drawing exactly 4.5 mL blood in 0.5 mL of 3.2% disodium citrate are preferred.
- All the glassware should be clean and dry.

Quick's One Stage Prothrombin Time

Principle: Prothrombin time (PT) is the time taken by citrated plasma to clot after the addition of tissue thromboplastin and calcium. It tests ***extrinsic and common pathway*** of coagulation system.

Reagents Required

- Thromboplastin reagent
- Test plasma: Centrifuge the patient's blood sample at 4500 RPM for 15 minutes and collect the platelet–poor plasma in a clean test tube.
- Control plasma from a normal person.
- Calcium chloride solution
- Water-bath at 37°C
- Stopwatch or a coagulometer.

Procedure

- Incubate all the reagents and test tubes at 37°C for 15 minutes.
- Pipette 0.1 mL of plasma into a test tube and add 0.1 mL of thromboplastin suspension. Incubate the tube at 37°C water-bath.
- Add 0.1 mL of calcium chloride solution to the tube after 1 minute and mix.
- Immediately start the stop watch and leave the tube in water-bath for a minimum of 8 to 9 seconds.
- Gently tilt the tube and look for clot. As soon as the clot appears, record the time.
- The same procedure is repeated for the control sample.

Normal Range: 11-16 Seconds

Reporting of Prothrombin Time

Prothrombin time may be reported in different ways:

- Patient PT and control PT in seconds
- Ratio of patient PT to control PT
- International normalized ratio (INR): Due to the inherent variation in the sensitivity of thromboplastin reagents, it is advisable to report the PT in international normalized ratio (INR). It provides a uniform scale in spite of using different sources of thromboplastin. WHO recommended that each thromboplastin should have ISI [International sensitivity index) value. INR is calculated using the formula:
  INR = (PT patient in seconds/ PT normal plasma in seconds) ISI

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Uses

- **Screening test to evaluate coagulation disorders:** It measures coagulation factor I, II, V, VII and X. Deficiency of any one of these factors leads to prolongation of PT. It should be used along with PTT.
- **To monitor oral anticoagulant therapy.**
- **To evaluate liver function:** Liver disease can result in deficiency of the coagulation factors. Hence PT should be performed before a *liver biopsy* and *prolonged* PT is a contraindication for liver biopsy.

Interpretation: **Prolonged PT is seen in:**

- Liver disease
- Administration of oral anticoagulants like coumarin
- Vitamin K deficiency
  - Obstructive jaundice
  - Hemorrhagic disease of the newborn
- Deficiency of factors I, II, V, VII and X
- Disseminated intravascular coagulation (DIC)

Precautions

- The ratio of anticoagulant to blood must be 1:9.
- Avoid hemolyzed and clotted blood samples.
- Test should be performed within 2 hours of collection.
- Correction for PCV must be done.
- Test should be done at 37°C.
- Always run a control sample with the patient sample.

Prothrombin time can also be measured by the instrument called coagulometer. Semi-automated and fully automated coagulometers are now available in the market. These coagulometers should be properly calibrated and monitored regularly.

Activated Partial Thromboplastin Time (Partial Thromboplastin Time)

Principle: Activated partial thromboplastin time (APTT) is the time taken for citrated plasma to clot in the presence of a surface activator (kaolin), phospholipid and calcium. Partial thromboplastin time (PTT) is a measure of the **intrinsic** and **common coagulation pathways.**

Reagents Required

- Citrated platelet poor plasma of the patient
- Platelet substitute-commercially available phospholipid
- Surface activator (kaolin)
- Calcium chloride
- Normal control plasma

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Procedure

- Pipette out 0.1 mL of plasma into a glass tube.
- Add 0.1 mL of thoroughly mixed kaolin suspension to plasma.
- Place plasma kaolin mixture in a water-bath at 37°C for 10 minutes.
- Mix 2 mL of platelet substitute suspension (phospholipid) with 2 mL of calcium chloride solution and incubate in the water-bath at 37°C.
- Deliver 0.2 mL of phospholipid-calcium chloride mixture into plasma-kaolin mixture and start the stop watch.
- Time taken for the mixture to clot is recorded.
- Record the APTT time for the control sample.
- Repeat the test with patient and control plasma. Time should be within 1 second of the previous reading.
  The test can be carried out on an automated coagulometer.

Normal Range: 30-40 Seconds

Reporting: The patient's value is always to be reported with the control values in seconds. A prolongation of the patient value more than 8 seconds of the control value is considered as abnormal.

***Precautions*:** Similar to that for prothrombin time.

Uses

- Best single screening test for coagulation disorders. This test is abnormal with deficiencies of II, V, VIII, IX, X, XI and XII.
- For screening—hemophilia A and B
- For detecting coagulation inhibitors
- For monitoring anticoagulant therapy like heparin.

Interpretation

Common causes of a prolonged **APTT** are:

- Inherited coagulation disorders—deficiency of factor II, V, VIII, I X, X, XI, XII (e.g. hemophilia A, hemophilia B)
- von Willebrand disease
- Disseminated intravascular coagulation
- Liver disease
- Heparin therapy
- Vitamin K deficiency
- Oral anticoagulant therapy

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Thrombin Time (TT)

Principle: This is the time taken for the citrated plasma to clot after the addition of commercial thrombin in the presence of calcium.

Reagents Required

- Citrated platelet poor plasma of the patient
- Thrombin- commercial with calcium chloride
- Control plasma.

Procedure

- Pipette 0.2 mL of test and control plasma in two separate tubes.
- Incubate in a water-bath at 37°C for 1 minute.
- Add 0.1 mL of thrombin reagent to each tube and start the stop watch.
- Observe the time when the clot is formed.

***Normal value*: 15-19 seconds.** Patient's TT should be within 2 seconds of control.

Use

- To detect decreased or abnormal fibrinogen

Interpretation: Increased in
- Hypofibrinogenemia
- DIC.

TESTS FOR FIBRINOLYTIC ACTIVITY

- ***Whole blood clot lysis:*** Normal blood clots do not dissolve in 24 hours. When there is increased fibrinolytic activity, the clot undergoes lysis within 30 to 60 minutes after clotting.
- ***Euglobulin lysis time:*** This is a quantitative measure of fibrinolytic activity. Euglobulin fraction of plasma includes plasminogen, plasminogen activators and fibrinogen. Lysis normally occurs in 2 to 4 hours. Values less than 2 hours indicate increased fibrinolysis.
- ***Fibrin degradation products:*** Serum/plasma of the patient is collected and latex particles coated with specific antibodies to purified fibrin degradation products (FDP) are added. Presence of agglutination suggests the presence of FDP in the serum. Semiquantitation of the FDP levels can be assessed by using different dilutions of the patient's serum.
  Use: Aids in diagnosis of DIC.
- ***D-dimer:*** D-dimer is a specific marker of DIC than FDP.
  Summary of screening tests for bleeding disorders are presented in Table 48.3.

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Table 48.3: Summary of screening tests for bleeding disorders

Investigation	*Normal range*	*Main causes of abnormal test*
Platelet count	150-450 × 10^9/L	Thrombocytopenia
Bleeding time (template method)	2-9 minutes	Thrombocytopenia Abnormal platelet function Deficiency of von Willebrand factor Vascular abnormalities
Prothrombin time (PT)	11-16 seconds	Deficiency of factor II, V, VII or X
Activated plasma thromboplastin time (APTT)	30-40 seconds	Deficiency of factor II, V, VIII, IX, X, XI, XII Heparin Antibodies against clotting factors Lupus anticoagulant
Thrombin time	15-19 seconds	Hypofibrinogenemia, DIC

SUMMARY

- Laboratory tests for bleeding disorders can be divided into screening tests, accessory tests and other specific tests.
- Platelet count and platelet aggregation tests are done for quantitative and qualitative assessment of platelets.
- Prothrombin time and activated partial thromboplastin time are the common screening tests. PT tests extrinsic and common pathway of coagulation system, whereas APTT tests intrinsic and common pathway of coagulation system.
- Tests for fibrinolytic activity mainly include assays for FDPs and D-dimer.

SELF-ASSESSMENT EXERCISES

I. Short Notes

1. Definition and methods of clotting time
2. Bleeding time
3. Prothrombin time
4. APTT
5. Thrombin time

II. Multiple Choice Questions

1. Prolonged bleeding time is found in all the conditions EXCEPT

A. Thrombocytopenia
B. von Willebrand disease
C. Aspirin intake
D. Hemophilia

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2. Best laboratory test for assessing platelet function is
A. Bleeding time
B. Clotting time
C. Clot retraction time
D. Prothrombin time

3. Hess test is NOT positive in
A. Vascular purpura
B. Defective platelet function
C. Thrombocytopenia
D. Hemophilia

4. Test useful for monitoring heparin therapy is
A. APTT
B. Prothrombin time
C. Bleeding time
D. Hess test

5. Laboratory test done to monitor oral anticoagulant therapy is
A. Prothrombin time
B. Hess test
C. Bleeding time
D. Clotting time

6. Prolonged PT is NOT seen in
A. Patient on oral anticoagulants like coumarin
B. Hepatitis A
C. Hemorrhagic disease of the newborn
D. Disseminated intravascular coagulation

7. Prolonged APTT is NOT observed in
A. Hemophilia A
B. Hemophilia B
C. von Willebrand disease
D. Immune thrombocytopenic purpura

8. Thrombin time is prolonged in
A. Hemophilia
B. Afibrinogenemia
C. Factor IX deficiency
D. Factor XII deficiency

ANSWERS

1. D **2.** A **3.** D **4.** A **5.** A **6.** B **7.** D **8.** B

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CHAPTER 49

Flow Cytometry

CHAPTER OUTLINE

- ❖ Introduction
- ❖ Applications of Flow Cytometry

INTRODUCTION

Flow cytometry (FCM) is a powerful, rapid and cost effective analytic technique in which cell suspension is stained with fluorescent labeled antibodies and subjected to analysis on a specialized instrument called flow cytometer.

Uses: FCM is useful for identifying and counting single cells labeled by fluorochrome monoclonal antibodies to specific surface or intracellular antigens. It provides detailed multiple intrinsic parameters of cells of normal, reactive, benign and neoplastic tissue. Thus, it can detect the:

- Cell lineage (e.g. B lymphocyte versus T lymphocyte).
- Degree of maturation (e.g. pre-B or mature B cells).
- Cell function (presence of cytokines or other receptors).

Specimen

- Fresh, unfixed tissue specimen from which single cell suspension can be generated is suitable for flow cytometric analysis.
- The cell suspension may be peripheral blood, bone marrow aspirates and core biopsies, fine-needle aspirates, fresh tissue biopsies (e.g., lymphnode) and body fluids.

Components of FCM (Fig. 49.1)

It is a relatively complex device with four major components.

- ***Fluidics system***
  - **Specimen stream**: Specimen is subjected to form a flowing stream of fluid such that the suspended cells move as single cell suspension in the central column.
  - **Sheath fluid**: Specimen stream is surrounded by buffered saline known as sheath fluid which maintains the monolayer of cells in the center of the flowing stream. This stream of single cells pass through the laser light.

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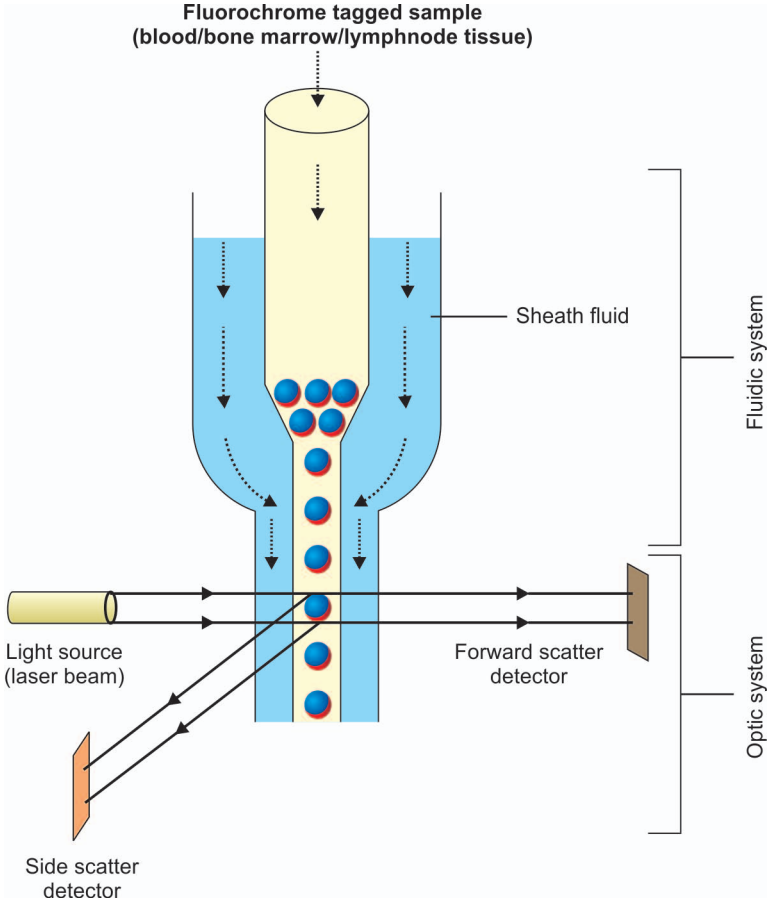


Fig. 49.1: Flow cytometer showing the fluidic and optic system

- *Optics system (Optics and light detectors)*
  - **Light source:**
    - The light source (usually lasers) illuminates the fluorochrome tagged cells which are propelled through the flow chamber.
    - The light hitting the cells is either scattered or absorbed and then re-emitted (fluorescence).
    - The pattern of scattered or absorbed laser light is dependent on cell size, shape, volume and granularity. Thus, it gives information about the intrinsic cellular parameters.
  - **Detectors:**
    - The light scattered or emitted by the cells as cells flow through the laser beam is measured by detectors. In addition, fluorescent molecules or fluorochromes attached to the cells emit fluorescence.

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  - Detectors convert the detected light into electrical impulses, the magnitude of which are proportional to the amount of light and gives information about the cross-sectional area of the cell.
- ***Electronic system*** measures electrical impulses and converts it into digital information that is gathered and interpreted by the analysis software. These profiles of cells are normally displayed as dot plots or histograms.
- ***Computer*** which controls the functions of flow cytometer.

APPLICATIONS OF FLOW CYTOMETRY

Flow cytometry is used in a variety of different fields including immunology, pathology and medicine. Common applications are:

- ***Diagnosis and classification***
  - Classification of hematopoietic neoplasms: Leukemia, lymphoma, myelodysplasia and systemic mastocytosis.
  - Diagnosis of PNH
- ***Prognosis***
  - Prognosis of hematopoietic neoplasms (e.g. chronic lymphocytic leukemia, acute leukemia)
  - Prognosis and stage of HIV**:** Enumeration of lymphocytes subset e.g. CD4+ T-cells as substitute marker for disease progression in AIDS.
- ***Monitor effect of therapy***
  - Therapeutic targets: FCM can identify potential therapeutic targets which is helpful mainly in hematopoietic neoplasms.
  - Detect multiple drug resistance in cancer chemotherapy.
- ***Detect minimal residual disease*** (e.g. leukemias)
- ***Other uses***
  - Transplantation: Histocompatibility cross match of organs for transplantation and in stem cell transplantation.
  - Enumeration of reticulocytes and platelets.
  - Detection of antibodies to platelets and neutrophils.
  - Detection of intracellular parasites (e.g. plasmodium, trypanosomes, babesia).

SUMMARY

- Flow cytometry (FCM) is a rapid analytic technique.
- It detects the cell lineage, degree of maturation of cells and cell function (presence of cytokines or other receptors).
- FCM is used for diagnosis and classification of hematopoietic disorders. It is also useful in assessing prognosis, monitor therapy and to detect minimal residual disease.

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SELF-ASSESSMENT EXERCISE

Multiple Choice Question

1. Flow cytometry is useful in hematology for the following conditions EXCEPT

A. Classification of leukemia
B. Classification of lymphoma
C. Diagnosis of PNH
D. Detection of HbA

ANSWER

1. D

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CHAPTER 50

Urine Analysis

CHAPTER OUTLINE

- Introduction
- Physical Examination
- Chemical Examination
- Microscopic Examination

INTRODUCTION

Urine analysis (urinalysis) reflects the state of function of the kidneys and urinary tract. It provides information about metabolic or systemic (nonrenal) disorders. Urine examination should precede all other invasive/non-invasive diagnostic investigations for renal function.

Collection of Urine Specimen

Urine should be collected in a clean, dry and preferably sterilized container. For routine examination, the first-morning (8 hours concentrated) sample is preferred but any fresh random urine specimen is satisfactory for chemical analysis. Demonstration of casts and crystals is easy with first morning samples.

Preservation of Urine

Urine sample should be **examined within 2 hours** of collection. If delay is likely to occur, it should be preserved by one of the following methods:

- Refrigeration without any preservative
- Use of preservatives like toluene, concentrated HCl, thymol and formaldehyde.

Examination of Urine

Urine examination consists of: (1) physical examination, (2) chemical examination and (3) microscopic examination.

PHYSICAL EXAMINATION

Physical properties of normal urine are shown in Table 50.1.

Volume

A healthy adult excretes about 600 to 2000 mL. of urine in 24 hours. In infants, the volume is 300-600 mL/ day. Volume is measured by collecting 24-hour urine samples in a measuring cylinder.

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Table 50.1: Physical properties of normal urine

Physical properties	*Normal urine*
Volume	600 to 2000 mL/day
Color	Yellow (straw to amber)
Transparency	Clear
Odor	Faintly aromatic
pH	4.6-8
Specific gravity	1.003-1.035 (adult-random urine)

Polyuria

Increased urine output (more than 2 liters in 24 hours).

Causes

- **Physiological:** Increased fluid intake
- **Pathological**
  - Diabetes mellitus (polydipsia—excessive intake of water)
  - Chronic renal diseases
  - Diuretic therapy
  - Diabetes insipidus
  - Primary aldosteronism.

Nocturia

Excretion of more than 500 mL of urine at night.

Oliguria

Decreased urinary output (less than 500 mL in 24 hours).

Causes

- Restricted intake of fluid
- Excessive loss of fluid, e.g. in hemorrhage, burns, dehydration and shock
- Renal diseases
  - Acute glomerulonephritis
  - Nephrotic syndrome
  - Acute tubular necrosis as in shock, burns, crush syndrome, incompatible blood transfusion and heavy metal poisoning
- Addison's disease.

Anuria

Markedly diminished urine output, usually less than 125 mL in 24 hours.

Cause

Renal ischemia, tumors and renal stones

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Color (Table 50.2)

Normal urine is **straw to amber colored** due to the presence of urochrome pigment, excretion of which is generally proportional to the metabolic rate.

Table 50.2: Conditions associated with color changes in urine

Color	*Condition*
Colorless	Dilute as in polyuria
Dark brown	Oliguria
Smoky (red or red-brown)	Red blood cells (hematuria)
Cola colored	Intravascular hemolysis
Yellow-brown	Bilirubin
Orange brown	Urobilin
Dark colored on standing	Alkaptonuria (presence of homogentisic acid)
Milky	Pus or chyle (chyluria)

Chyluria

Chyluria is a rare condition in which the **urine contains lymph.** Obstruction to lymph flow and rupture of lymphatic vessels into the renal pelvis, ureters, bladder or urethra may be associated with chyluria. The causes include filariasis, abdominal lymphadenopathy and tumors. The amount of lymph determines the color of urine which may range from clear to opaque or milky.

Lipiduria

In nephrotic syndrome, urine shows fat globules which are triglycerides (neutral fat) and cholesterol. It may also be observed in patients with bone fractures.

Transparency and Turbidity

Normal urine is usually clear when passed fresh. It may become cloudy, hazy or turbid with the presence of mucus, phosphates, pus, crystals, blood, casts or bacterial growth.

Odor (Table 50.3)

Normal urine has a faintly aromatic odor because of volatile acids.

Table 50.3: Causes of different odor

Odor	*Cause*
Fruity (sweet)	Presence of acetone (ketonuria)
Ammoniacal	Bacterial decomposition
Mousy	Phenylketonuria
Putrid or foul	Severe urinary tract infection

Reaction (pH)

Normal urine is usually acidic with pH varying from 4.6 to 8. Urinary pH is measured as given below.

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- **Litmus paper**
  Technique: The reaction of urine is determined with blue and red litmus paper. The urine when examined must be fresh as it turns alkaline on standing due to bacterial decomposition
  - **Acidic urine** turns blue litmus paper red
  - **Alkaline urine** turns red litmus paper blue
  - Both blue and red litmus papers turn reddish purple when the urine is faintly on the acid side (pH 7.0) or neutral.
- **pH indicator paper strips/strip multistix method** uses a methyl red and bromthymol blue double indicator system which can measure a pH from 5 to 9.

Cause

Causes of alkaline and acidic urine are shown in Table 50.4

Table 50.4: Causes of alkaline and acidic urine

Alkaline urine	*Acidic urine*
• Metabolic and respiratory alkalosis	• High protein diet
• Phosphaturia	• Febrile illness
• Severe vomiting	• Ketonuria
• Infection with ammonia producing urea splitting bacteria like proteus	• Leukemia
• Potassium deficiency	• Starvation
• Hyperaldosteronism	• Severe diarrhea
• Intake of fruits of the citrus family (orange)	• Diabetes mellitus
• Intake of bicarbonates	• Respiratory disease

Specific Gravity

Specific gravity depends on the number, density and weight of the solute particles in the urine. It is used as a measure of the concentrating power of the kidney. The specific gravity of urine is its density compared with the density of distilled water that is conveniently fixed as 1.000 at 20°C. Normal specific gravity of a 24-hour urine sample is 1.003 to 1.035, average being 1.016. Specific gravity (SG) is measured by: (1) urinometer, (2) refractometer and, (3) dipstick method.

Urinometer and refractometer methods are more accurate as compared to the dipstick method.

Urinometer Method

Urinometer (Fig. 50.1) is a specialized hydrometer. It consists of a weighted glass cylinder which floats in urine and has a calibrated stem to measure specific gravity at a given temperature. The scale in many urinometers read in small divisions from 1.000 to 1060. It is usually calibrated in the range of 15° to 20°C (depends on the manufacturer). As the specific gravity varies with temperature, the reading obtained should be corrected to the room temperature by applying temperature correction.

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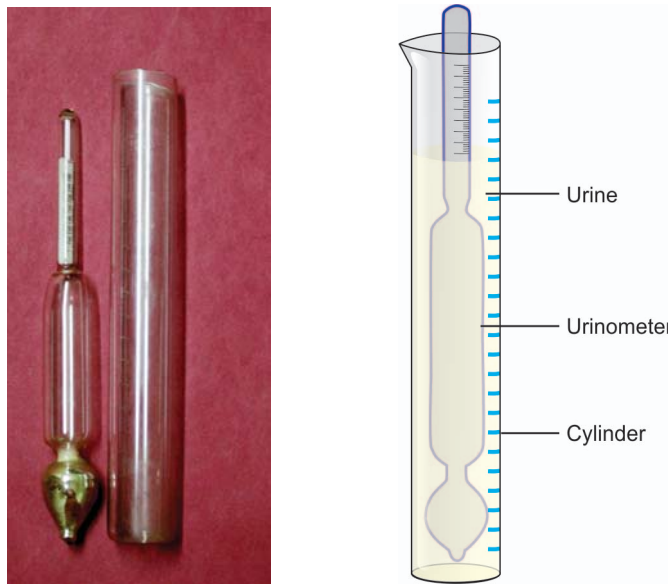


Fig. 50.1: Urinometer for specific gravity measurement

- **Temperature correction:** For **every 3°C rise** in room temperature beyond the calibrated temperature (15°C), **add 0.001** to the recorded reading; and **subtract 0.001** for every 3°C fall in temperature.
- **Correction for protein and sugar:** Subtract 0.003 for every 1 g/dL of protein and 0.004 for every 1 g/dL of glucose.

Technique

The urine is poured into a wide necked vessel (cylinder), and the urinometer is floated so that it does not touch the sides of the container. The specific gravity is read from the graduations which lie at level with the true surface of the urine (lower meniscus). Observe the temperature of the urine. Check the temperature at which the urinometer is calibrated.

- **Technique when only small volumes of urine are available:** The urine may be diluted with equal or double its volume of distilled water.

  The specific gravity is calculated as follows:
  - **Dilution with equal volume** = Last two figures of the observed value × 2
  - **Dilution with double volume** = Last two figures of the observed value × 3.

Refractometer Method

It is based on refractive index of urine and has the advantage of requiring only a few drops of urine.

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Dipstick Method

SG is recorded by change of color on the strip and compared with the color chart on the multistix bottle.

Principle

It is an indirect method for measuring specific gravity. The reagent area of dipstick has three main ingredients namely: polyelectrolyte, indicator substance and buffer. This method is based on the pK_a (symbol for the quantitative measure of the strength of an acid in solution) change of the pretreated polyelectrolytes in relation to ionic concentration of the urine. When the ionic concentration is high, the pK_a as well as pH is decreased. The indicator substance (bromothymol blue) then changes its color relative to ionic concentration and this is translated to specific gravity values. The value is not affected by high amounts of glucose, protein or radiographic contrast material in urine.

Causes

- **Increased** specific gravity
  - Glycosuria
  - Dehydration: Restricted fluid intake, diarrhea, vomiting, fever, and excessive sweating.
- **Decreased** specific gravity
  - Excessive fluid intake
  - Diabetes insipidus
  - End stage kidney
    - Chronic glomerulonephritis
    - Chronic pyelonephritis
    - Bilateral polycystic kidneys
    - Hypertension
- **Low and fixed** specific gravity: When **specific gravity is fixed** at 1.010, this is known as **isosthenuria**. It is indicative of severe renal damage (chronic renal failure) with disturbance of both the concentrating and diluting abilities of the kidney.

Interpretation

- Specific gravity provides information about the renal status and hydration.
- It depends on the nature and amount of the dissolved constituents. Urine concentration and dilution is modulated by the tubular absorptive and secretory functions thereby affecting the specific gravity.

CHEMICAL EXAMINATION

Chemical constituents of a normal urine sample are shown in Table 50.5.

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Table 50.5: Chemical constituents in normal urine	
Chemical substance	*Normal urine*
Protein, albumin	Negative-trace
Glucose	Negative
Ketone bodies	Negative
Bilirubin	Negative
Urobilinogen	Trace
Bile salts	Negative
Blood	Negative

Proteinuria

The presence of detectable protein in the urine is known as **proteinuria**. It indicates glomerular injury. If turbid, filter or centrifuge the urine before testing.

Tests for protein

a. Heat and acetic acid test
b. Sulfosalicylic acid test
c. Heller's test
d. Dipstick method

Heat and Acetic Acid Test

- ***Principle:*** Heat induced **coagulation of proteins** and precipitation. Coagulation can be further enhanced when drops of acetic acid are added.
- ***Procedure*** **(Fig. 50.2):**

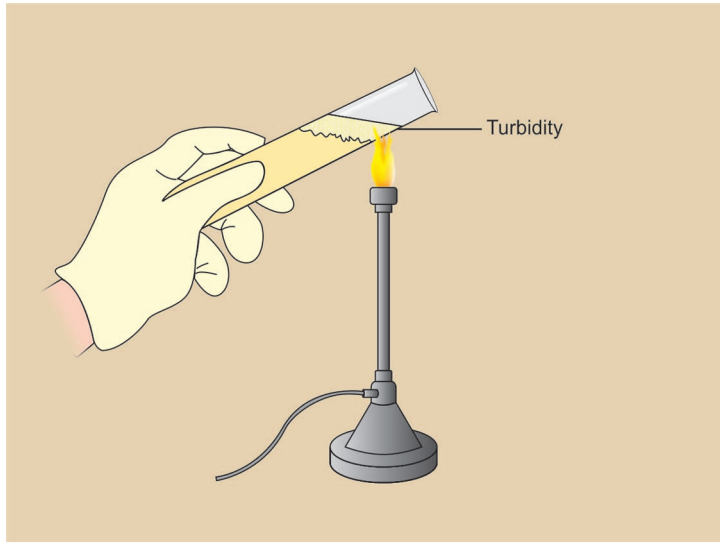


Fig. 50.2: Method of performing heat and acetic acid test

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- Fill three-fourth of a test tube with clear urine.
- **Heat the upper part (1/3) of the urine** (the lower part of the urine acts as a control for checking turbidity in the heated upper part). **Do not boil the urine**, as it causes mixing of the upper and lower parts of urine and traces of proteins cannot be appreciated. The development of **turbidity may be due to coagulation of proteins or due to phosphates.**
- Add a few (3-5) drops of 10% glacial acetic acid and if turbidity persists it is due to proteins (phosphates will dissolve).

- ***Interpretation:*** Depending on the amount of precipitate, results are interpreted as shown in Table 50.6.

Table 50.6: Interpretation of heat and acetic acid test

Description	*Result*	*Approximate protein concentration*
No cloudiness/precipitate	Negative	-
White cloudiness	+	<100 mg/dL
Granular white precipitate	++	100 to 250 mg/dL
Floccular precipitate	+++	250 to 500 mg/dL
Thick opaque precipitate	++++	500 mg/dL

- ***Precaution:*** Before testing for proteinuria, the pH of the urine should be checked. If alkaline, the urine should be acidified with a few drops of glacial acetic acid.

Sulfosalicylic Acid Test

This test **detects all types of proteins** (albumin, globulin, glycoproteins and Bence Jones proteins).

- ***Principle:*** **Cold precipitation of proteins** by a strong acid.
- ***Procedure***
  - Take 2.5 mL of urine in a small test tube.
  - Slowly pour 2.5 mL of sulfosalicylic acid. Wait for 5 minutes.
- ***Interpretation (Table 50.7):*** Presence of a **cloudy precipitate** indicates the presence of proteins in urine. However, it also precipitates mucus and Bence Jones proteins.
- ***Precaution:*** Filter urine if turbid, as it interferes with the final reading.

Table 50.7: Interpretation of sulfosalicylic acid test

Description	*Result*	*Approximate protein concentration*
Negative	No turbidity	< 5 mg/dL
Barely perceptible turbidity	Trace	5 to 20 mg/dL
Distinct turbidity without granulation	+	30 mg/dL
Turbidity with granulation, no flocculation	++	100 mg/dL
Turbidity with granulation and flocculation	+++	300 to 500 mg/dL
Clumps of precipitated protein	++++	> 500 mg/dL

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Heller's Test

- ***Principle:*** **Cold precipitation of proteins** by a strong acid.
- ***Procedure:*** Take 5 mL of urine in a test tube and add a few drops of concentrated nitric acid.
- ***Interpretation:*** Presence of proteins is indicated by a **white ring** at the junction of urine and acid.
- ***Precaution:*** Filter urine if turbid as it interferes with the final reading.

Dipstick Method

- ***Principle:*** The test is based on the protein error of pH indicators. At a constant pH any color change in the indicator is due to protein. The reagent strip is impregnated with an indicator tetrabromophenol blue or tetrachlorophenol-tetrabromosulfophthalein buffered to pH 3.0. At this pH it is yellow in the absence of protein. If urine contains protein (which will elicit a pH change), it forms a complex with the indicator turning its color to green or bluish green.
- ***Procedure***
  - The reagent area of the strip is completely immersed in fresh urine for 1-2 seconds. It is taken out and excess urine is drained off by gently tapping the edge of the strip against the side of the urine container.
  - Change in the color of the strip is compared with the reference color chart for proteins on the dipstick container. Strips are sensitive and able to detect as low as 20 mg/dL of albumin in the urine.
- ***Interpretation:*** The strip is yellow in the absence of protein and variable shades of green develop depending on the type and concentration of protein present.

Quantitative Estimation of Proteins in Urine

Protein excretion in a 24-hour urine sample is required in suspected cases of nephrotic syndrome (>3.5 gm/24 hours) and orthostatic/postural proteinuria. This may be carried out by **Esbach's albuminometer** (Fig. 50.3) **method.**

- ***Principle:*** Cold precipitation of proteins by a strong acid.
- ***Procedure***
  - Fill the albuminometer with urine up to the mark U.
  - Add Esbach's reagent up to the mark R.
  - Stopper the Esbach's albuminometer, mix and allow it to stand for 24 hours.
  - Take the reading from the level of precipitation in the albuminometer and divide the value by 10 to get the percentage of total proteins.
- ***Precaution:*** The urine must be of acid reaction, clear and should not be concentrated, in which case it must be diluted with water making allowance for the dilution in the final calculation.

Causes of Proteinuria (albuminuria)

Proteinuria (Table 50.8) may be classified as **glomerular, tubular, lower urinary tract, prerenal and asymptomatic.** It may also be classified depending on the amount of protein as **heavy** (> 4 gm/day), **moderate** (1 to 4 gm/day) and **mild** (less than 1 gm/day) proteinuria.

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Fig. 50.3: Esbach's albuminometer used for quantitative measurement of proteins in urine

Table 50.8: Causes of proteinuria

Causes of proteinuria	*Amount of proteinuria*
Glomerular damage	
• Nephrotic syndrome	Heavy
• Acute glomerulonephritis	Mild-to-moderate
• Acute rapidly progressive glomerulonephritis	Moderate-to-heavy
• Chronic glomerulonephritis	Moderate-to-heavy
Tubular damage	
• Pyelonephritis	Mild
• Acute tubular necrosis	Mild
• Polycystic kidney disease	Mild
• Heavy metal and vitamin D intoxication	Moderate
• Hypokalemia	Mild
Prerenal	
• Multiple myeloma (Bence Jones protein).	Moderate
• Nephrosclerosis	Mild-to-moderate
• Malignant hypertension	Moderate-to-heavy
• Systemic lupus erythematosus	Moderate-to-heavy
• Congestive heart failure	Mild
• Dehydration	Mild
Asymptomatic proteinuria	
• After excessive exercise	Mild
• Postural (orthostatic) albuminuria	Mild

- **Postural (orthostatic) albuminuria:** In this condition, protein is seen in the urine only when the patient is active on his feet. At night when recumbent, the early morning sample is negative for protein. It may be related to renal congestion and ischemia when patient is active.

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- **Bence Jones proteins:** Bence Jones proteins are light chains of immunoglobulins, secreted in **multiple myeloma.** It may also be found in macroglobulinemia and lymphoma. Bence Jones proteins precipitate at temperature between 40°C and 60°C, and redissolve near 100°C. It reappears on cooling to 40°-60°C.

  Acetic Acid Test for Bence Jones Protein
  - ***Procedure***
    - Take 5 mL of clear urine in a test tube and 1 mL of acetate buffer.
    - Heat the urine in a water-bath with a thermometer inside.
    - At 40°C, Bence Jones proteins start precipitating till 58°C when the precipitation is complete.
    - If urine is heated beyond 60°C, Bence Jones proteins dissolve in the urine and reappear on cooling at 60°C.

      However, the best method for detection of Bence Jones protein in urine is by protein electrophoresis.
- **Combined albuminuria with Bence Jones proteinuria:** In cases where both albumin and Bence Jones proteins are present, boil the urine and filter. Precipitated albumin will be filtered out. Take the filtrate and heat it to 40°-60°C when Bence Jones proteins precipitate. These dissolve on boiling.
- **Microalbuminuria:** Screening tests for urine protein are not sensitive enough to detect very small quantities of protein. Microalbuminuria is the presence of albumin in urine above the normal level but below the detectable range of conventional methods. It is defined as the **persistent elevation of the urinary albumin excretion of 20-200 mg/L** (or 20-200 micrograms/min) in an early morning urine sample. It indicates early and possibly reversible glomerular damage.

  Causes of microalbuminuria
  - **Diabetes mellitus:** In diabetic patients, presence of microalbuminuria is associated with **increased cardiovascular mortality** and is a risk factor for **renal mortality.** Early detection can predict the development of renal complications in diabetics.
  - **Essential hypertension:** In hypertensive patients, microalbuminuria **predicts cardiovascular morbidity and mortality.**

Reducing Substances in Urine

Reducing substances are those compounds which reduce cupric ions (from copper sulfate in Benedict's reagent) in an alkaline solution to cuprous ions (cuprous oxide). Such substances may be sugar or nonsugar.

Sugar: These include: glucose, fructose, pentose, galactose, lactose, maltose and sucrose. Special tests are required to differentiate the sugar occurring in urine.

Nonsugar: Ascorbic acid, uric acid, urates, glucuronides, chloroform, formaldehyde, salicylates, streptomycin, phenol, PAS, homogentisic acid (alkaptonuria) and creatinine.

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Blood glucose level varies between 70 to 120 mg/dL. This may increase to 120 to 160 mg/dL after a meal. Normally, all the glucose in the blood is filtered through glomerulus and reabsorbed at proximal tubules. If the renal threshold (the lowest blood glucose level that will result in glycosuria) is exceeded (usually greater than 180 to 200 mg/dL), the excess glucose will not be reabsorbed into the blood and will be eliminated in the urine as in cases of diabetes mellitus. The presence of detectable amounts of **glucose in urine** is termed **glycosuria**.

Tests for reducing substance — sugar and nonsugar

a. **Benedict qualitative test**
b. **Dipstick method**

Benedict Qualitative test (semiquantitative)

This test detects the presence of reducing substances in urine and is not specific for glucose.

- ***Principle:*** The copper sulphate present in the Benedict's reagent reacts with the **reducing substances** in the urine which **convert cupric sulphate to cuprous oxide** in hot alkaline media. Thus, this test is based on the reduction of cupric ions in Benedict's solution to cuprous ions. In the absence of reducing substances in urine, the color of the reagent remains blue.
- ***Procedure*** **(Fig. 50.4)**
  - Take **5 mL of Benedict's** (qualitative) reagent in a test tube.
  - Boil to exclude presence of reducing substance in reagent.
  - Add **8 drops (0.5 mL) of protein free urine.** Boil the mixture for 5 minutes and allow to cool. The **ratio** of 5 mL Benedict's reagent and 8 drops (0.5 mL) of urine **ratio is important because it is a semiquantitative test.**
  - Note the color of the precipitate which is cuprous oxide formed due to reduction of cupric sulphate of Benedict's reagent.
- ***Interpretation:*** The change of color from blue to green, yellow, and orange/red depends on the amount of sugar present (Fig. 50.4).
- ***Precaution:*** If albumin is present in any considerable quantity, it interferes with reduction of copper in the copper tests and should be removed by acidifying with acetic acid, boiling and filtering.

Dipstick Method

- ***Principle:*** Diastix/multistix/dipstix contains: (i) glucose oxidase (ii) peroxidase (iii) chromogen: O-toluidine (clinistix), potassium iodide (multistix/diastix).

  Glucose present in the urine is oxidized by atmospheric oxygen in the presence of glucose oxidase to gluconic acid and hydrogen peroxide. The hydrogen peroxide, in the presence of peroxidase, oxidizes the reduced chromogen present in the dipstick to various shades of purple (oxidized chromogen). The color change depend on the amount of glucose (semi-quantitative) present in the urine.

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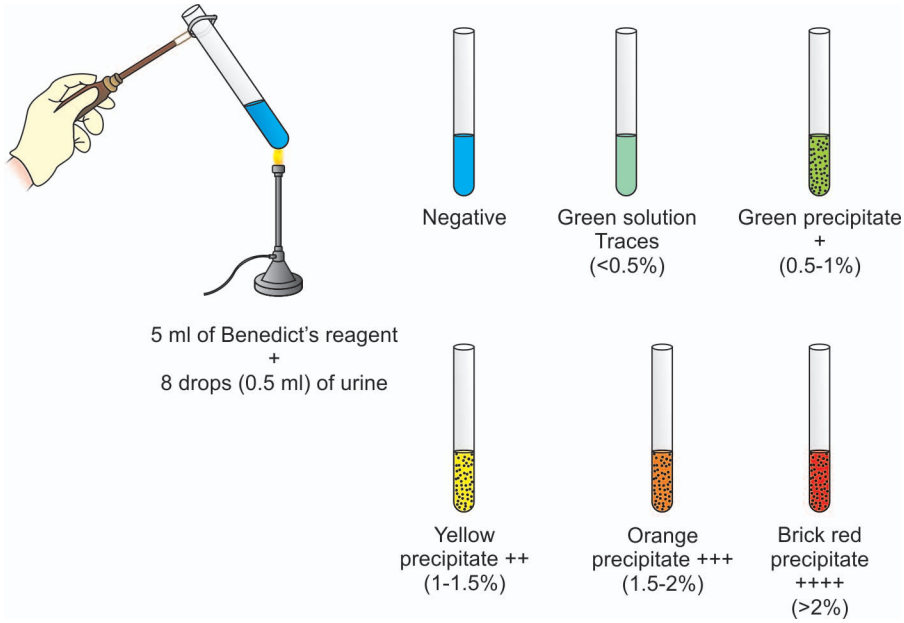


Fig. 50.4: Method and interpretation of Benedict's test

Step 1:

$$\text{Glucose (in urine)} + \text{oxygen (from air)} \xrightarrow{\text{Glucose oxidase}} \text{Gluconic acid} + H_2O_2$$

Step 2:

$$H_2O_2 + \text{reduced chromogen} \xrightarrow{\text{Peroxidase}} \text{Oxidized chromogen} + H_2O$$

- ***Procedure***
  - The reagent area of the strip is completely immersed in fresh urine for 1-2 seconds. It is taken out and excess urine is drained off.
  - The change in color of the test area is compared with the color chart on the dipstick container exactly after 30 seconds (or as per manufacturer) after dipping the strip in the urine.

Cause of Glycosuria (Table 50.9 and Chapter 57)

The substances which may give positive reaction with Benedict's test but negative with glucose oxidase strips are shown in Table 50.10.

Fructosuria: Presence of fructose in urine. It is detected by Seliwanoff's test

Causes: High intake of fruits like grapes and oranges.

Lactosuria: Presence of lactose in urine. It is detected by Rubner's test

Causes: Suckling infants and nursing mothers

Pentosuria: Presence of pentose in urine. It is detected by Bial's test

Causes: Ingestion of fruits

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Table 50.9: Causes of glycosuria

- Diabetes mellitus
- Pregnancy
- Renal glycosuria
- Alimentary glycosuria
- Endocrine disorders
  - Acromegaly
  - Cushings syndrome
  - Hyperadrenocorticism
  - Functioning α- or β-cell pancreatic tumors
  - Hyperthyroidism
  - Pheochromocytoma
- Increased intracranial tension (brain tumor or hemorrhage).

Table 50.10: Causes of false positivity by nonsugar reducing substances

- Ascorbic acid
- Glucuronic acid
- Homogentisic acid
- Salicylates
- Phenylketonuria
- Tyrosinemia

Ketone Bodies

The presence of ketone bodies in the urine is a measure of the metabolic rather than renal function. Whenever there is a defect in carbohydrate metabolism, the fat is used as a source of energy. When increased quantities of fat are metabolized, there is increased production of ketone bodies which begin to accumulate in the blood and are subsequently excreted in the urine.

Ketone bodies are three metabolically related compounds:

- Acetoacetic (diacetic) acid
- β-hydroxybutyric acid
- Acetone.
  If urine is left at room temperature, acetoacetic acid slowly converts into acetone.

Tests for ketone bodies

a. **Rothera's test**
b. **Gerhardt's test (ferric chloride test)**
c. **Hart's test**
d. **Dipstick method**

Rothera's Test

- ***Principle:*** **Acetoacetic acid** (diacetic acid) **and acetone react with sodium nitroprusside** in presence of an alkali to form a purple color compound.

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- ***Procedure***
  - Take 4 mL of urine in a test tube.
  - Add a few crystals of sodium nitroprusside and saturate the urine with ammonium sulphate by mixing vigorously.
  - Overlay with few drops of liquor ammonia along the wall of the tube.
- ***Interpretation:*** Development of a **purple ring** (Fig. 50.5) indicates the presence of **acetoacetic acid/acetone** or both. A brown or red color is of no significance.
- ***Precaution:*** Always test fresh urine as acetone evaporates on standing and bacterial growth causes loss of acetoacetic acid.

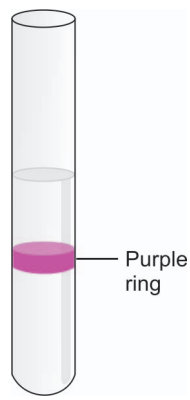


Fig. 50.5: Positive Rothera's test showing a purple ring

Gerhardt's Test (ferric chloride test)

This is neither a very specific nor a sensitive test. The test **detects acetoacetic acid.**

- ***Principle:*** **Acetoacetic acid reacts with ferric chloride** to give a deep red color.
- ***Procedure***
  - Take 8 mL of urine in a test tube.
  - Add 10% ferric chloride solution drop by drop; a precipitate of ferric phosphate forms.
  - Add more ferric chloride solution till the ferric phosphate precipitate disappears.
  - Filter it and add excess of ferric chloride to the filtrate.
- ***Interpretation:*** A **red color** of the filtrate indicates positive test.
- ***Precaution:*** The test should be carried out on fresh urine, since acetoacetic acid is converted to acetone, which does not give a positive reaction by this test. Salicylates and phenol when present in the urine strike a dark violet color with this reagent.

Hart's Test

This test detects **β-hydroxybutyric acid**, which the above two tests fail to detect. β-hydroxybutyric acid is usually accompanied by acetoacetic acid.

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- ***Principle:*** **β-hydroxybutyric acid is converted into acetone** which **reacts with sodium nitroprusside and liquor ammonia** to give purple red color.
- ***Procedure***
  - Take 20 mL of urine.
  - Add 20 mL distilled water. Boil till the solution reduces to half. This removes acetone and acetoacetic acid.
  - Take 10 mL of boiled, diluted urine and acidify with a few drops of glacial acetic acid.
  - Add 1 mL of hydrogen peroxide followed by 10 drops of sodium nitroprusside solution. Overlay with liquor ammonia along the side of the test tube.
- ***Interpretation:*** Purple color ring is formed.
- ***Precaution:*** Test to be done on fresh urine sample.

Dipstick Method

- ***Principle:*** Dipstick contains buffers and sodium nitroferricyanide, which react with acetoacetic acid in the urine to form a pink-maroon color in 15 seconds. The strips detect acetoacetic acid and not acetone. However, acetone can be detected if glycine is incorporated into it.
- ***Procedure:*** Dip the strip in urine, drain excess urine and read in 15 seconds.
- ***Precaution:*** The test is sensitive and it should be performed on fresh urine before acetoacetic acid decomposes to acetone.

Causes of ketonuria *(Table 50.11)*

Table 50.11: Causes of ketonuria

- Diabetic ketoacidosis
- Starvation
- Prolonged vomiting or diarrhea
- Prolonged febrile illness
- von Gierke's disease
- Eclampsia

Bilirubin (bile pigment)

Tests for bilirubin in urine provide information concerning metabolic or systemic disorders, especially liver function. Bilirubin is a breakdown product of hemoglobin and is normally not present in urine. Even trace amounts are clinically significant and only conjugated bilirubin is found in urine. Bilirubinuria causes yellow-brown to greenish-brown urine and forms yellow foam on shaking. Bilirubin is found only in freshly voided urine which upon standing is oxidized to biliverdin.

Tests for bilirubin

a. Fouchet's test
b. Dipstick method

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Fouchet's Test

- ***Principle:*** Fouchet's reagent contains **trichloroacetic acid and ferric chloride.** In an acidic medium ferric chloride oxidizes **bilirubin** to produce a dark green colored **biliverdin.**
- ***Procedure***
  - Take 10 mL of urine in a test tube and add 3 mL of 10% barium chloride solution.
  - Mix the two and filter the mixture through a filter paper. Bilirubin along with barium salt remains on filter paper.
  - Add a few drops of Fouchet's reagent onto the filter paper.
- ***Interpretation:*** **Green** or **blue color** (Fig. 50.6) indicates bilirubinuria.
- ***Precaution:*** Test to be done on fresh urine sample.

Note: Foam test is a simple test to detect bile pigments at the bed side. Take urine in a test tube and shake the urine. If bile pigments are present in the urine, the foam on top will be yellow in color.

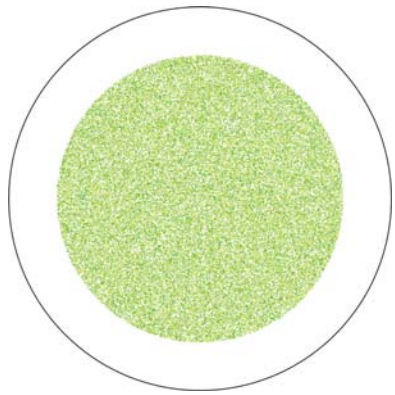


Fig. 50.6: Positive Fouchet's test showing greenish blue color

Dipstick Method

- ***Principle:*** The test is **based on a diazo reaction.** Bilirubin in the urine couples with a diazotized dichloronaniline (content of strip) in a strongly acidic medium to form colored compound namely azobilirubin.
- ***Procedure:*** Immerse the strip in urine, drain excess urine and read after specified time.
- ***Interpretation:*** A **positive** test indicates elevated conjugated serum bilirubin.
- ***Precaution:*** The tests are specific for bilirubin but the presence of other highly colored substances in urine may result in false positive tests.

Causes of Bilirubinuria

- Obstructive jaundice: Urine shows bilirubin without urobilinogen
- Hepatocellular jaundice: Bilirubin is **absent** in urine of patients with **hemolytic jaundice.**

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Urobilinogen

Urobilinogen is normally present in urine in trace amount (1-2 mg/dL) and is insufficient to cause a significant positive reaction. Whenever the liver is unable to efficiently remove the reabsorbed urobilinogen from the portal circulation (e.g. liver diseases, hemolytic anemia) more urobilinogen than normal is routed through the kidney and hence excreted in the urine.

Tests for urobilinogen

a. **Ehrlich's test**
b. **Dipstick method**

Ehrlich's Test

- ***Principle:*** Ehrlich's reagent reacts with urobilinogen and forms a pink colored aldehyde complex.
- ***Procedure***
  - Take 2.5 mL urine in a test tube.
  - Add 2.5 mL of Ehrlich's aldehyde reagent (paradimethyl amino benzaldehyde). Mix well by inversion.
  - Add 10 mL of saturated sodium acetate solution and mix.
- ***Interpretation:*** Pink to cherry red color indicates presence of urobilinogen.
- ***Precaution:*** This test should be carried out on freshly voided urine, since urobilinogen gets oxidized to urobilin; the conversion can be prevented by adding a few crystals of anhydrous sodium carbonate to urine.

Dipstick Method

- ***Principle:*** This method is based on the Ehrlich's test. The strip is coated with p-amino benzaldehyde with a color enhancer. It reacts with urobilinogen in a strongly acidic medium to produce a pink-red colored compound. The color is matched with the color chart on the container of the dipsticks.

Causes of Increased Urobilinogen in Urine

- Hemolytic anemias (without bilirubin in urine)
  - Thalassemia
  - Sickle cell anemia
  - Hereditary spherocytosis
- Liver diseases
  - Preicteric phase of infective hepatitis
  - Drugs or toxic hepatitis
  - Cirrhosis.

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Causes of Decreased/Absent Urobilinogen in Urine

In **obstructive jaundice,** bilirubin does not reach the intestine and hence is not converted into urobilinogen.

Bile Salts

Bile salts are composed of mixture of bile acids and glycine or taurine. Two important bile salts are sodium and potassium salts of glycocholates and taurocholates. Normally, bile salts are not present in urine.

Hay's Sulfur Test

- ***Principle:*** **Bile salts** have unusual property of **lowering the surface tension of urine** markedly even when present in small concentrations. This property is made use of in the Hay's test.
- ***Procedure:***
  - Take 10 mL urine in a wide bore test tube (2 cm diameter or more) or a small beaker.
  - Sprinkle sulfur powder over its surface, watch for 5 minutes.
- ***Interpretation:*** **Sulfur powder sinks to the bottom** of test tube (Fig. 50.7) in the presence of bile salts in urine.
- ***Precaution:*** Since soap can give false positive result, the test tube should be clean.

Causes: Hepatocellular and obstructive jaundice.

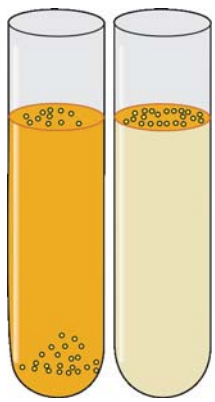


Fig. 50.7: Positive (left) and negative (right) Hay's test



Fig. 50.8: Positive Benzidine test

Tests for Blood in Urine

These tests detect hematuria, hemoglobinuria or myoglobinuria.

Hematuria: Presence of red cells in urine, e.g. renal stones, renal cell carcinoma.

Hemoglobinuria: Presence of free hemoglobin in urine, e.g. intravascular hemolysis.

Myoglobinuria: Presence of myoglobin in urine, e.g. crush injury to muscle, strenuous exercise.

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Tests for blood

a. **Benzidine test**
b. **Orthotoluidine test**
c. **Dipstick method**

Benzidine Test

- ***Principle:*** The test depends upon the ability of **heme compounds** derived from hemoglobin to **catalyze the oxidation of benzidine by hydrogen peroxide.**
- ***Procedure:***
  - Dissolve a small amount (knife-point full) of benzidine in 2 mL of glacial acetic acid and add an equal volume of 3% hydrogen peroxide.
  - From the above, take 2 mL in another test tube and add 2 mL of previously boiled and cooled urine and mix.
- ***Control for the test:*** If the test is negative, add a drop of blood to the above test tube. It should give blue color. This is to confirm the potency of reagents especially hydrogen peroxide and excludes a false negative reaction.
- ***Interpretation:*** The appearance of **blue color** (Fig. 50.8) indicates the presence of blood.
- ***Precaution:*** Presence of hypochlorite (bleach) and microbial peroxidase can cause false positive results. **Benzidine is carcinogenic**.

Orthotoluidine Test

- ***Principle:*** **Peroxidase-like activity of heme** present in hemoglobin **liberates oxygen from hydrogen peroxide** in the reagent. The liberated **oxygen changes the color of orthotoluidine.**
- ***Procedure:***
  - Take 2 mL of urine in a test tube.
  - Add 1 mL of orthotoluidine solution in glacial acetic acid.
  - Add few drops of hydrogen peroxide.
- ***Interpretation:*** The appearance of blue or green color indicates the presence of blood.

Dipstick Method

Very small amount of hemoglobin/RBCs are detected by dipstick method.

- ***Principle:*** The test is based on the **liberation of oxygen from peroxide in the reagent strip by peroxidase-like activity of heme** present in hemoglobin. The liberated oxygen changes the color of the chromogen. The reagent area is impregnated with organic peroxide and the chromogen is orthotoluidine/tetramethylbenzidine.

$$\text{Organic peroxide} \xrightarrow{\text{(peroxidase from Hb)}} H_2O + O$$

$$O + \text{chromogen} \xrightarrow{\text{(Orthotoluidine)}} \text{Oxidized chromogen (colored compound)}$$

- ***Procedure:***
  - Urine sample is mixed thoroughly so that sedimented RBCs are mixed.
  - Dipstick is dipped in urine for the time given. Color is matched.

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Causes of Hematuria

Urine is red colored in severe hematuria and smoky in mild hematuria. RBCs are demonstrable in urinary sediment. Causes of hematuria are shown in Table 50.12.

Table 50.12: Causes of hematuria

Renal diseases
- **Glomerular causes**
  - Acute glomerulonephritis
  - Renal infarct
  - Bacterial endocarditis with kidney involvement
  - Malignant hypertension
- **Nonglomerular causes**
  - Polycystic kidneys
  - Renal cell carcinoma
  - Renal stones (nephrolithiasis)
  - Trauma (including renal biopsy)
  - Renal tuberculosis
  - Hydronephrosis

Lower urinary tract/prostatic diseases
- Bladder stones (lithiasis)
- Cystitis
- Urethritis
- Carcinoma bladder
- Carcinoma prostate

Blood disorders
- Bleeding disorders: Coagulation disorders, severe thrombocytopenia
- Acute leukemia
- Sickle cell disease

Miscellaneous
- Instrumentation of urinary tract

Hemoglobinuria

Hemoglobin is too large to pass through the glomerular filter. If the renal threshold is exceeded the hemoglobin can pass into the urine which becomes cola colored.

- ***Procedure:*** Take 5 mL of urine and centrifuge at 1500-2000 RPM.
- ***Interpretation:*** If the supernatant is colored pink, it indicates hemoglobinuria.
  Note: Hemoglobin is precipitated when the urine is 80% saturated with ammonium sulphate but myoglobin is not.

Causes of hemoglobinuria (Table 50.13): Any cause of hemolysis may cause hemoglobinuria and the presence of hemoglobinuria indicates significant intravascular hemolysis.

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Table 50.13: Causes of hemoglobinuria

- Paroxysmal nocturnal hemoglobinuria (PNH)
- Paroxysmal cold hemoglobinuria (PCH)
- Incompatible blood transfusion
- Severe burns
- Autoimmune hemolytic anemia (AIHA), march hemoglobinuria
- Snake bite
- Blackwater fever (falciparum malaria)

Multistix Reagent Strips for Urine Testing

These have single/multiple discrete cellulose squares which are impregnated with reagents for testing glucose, protein, pH, specific gravity, hemoglobin, ketone bodies, bilirubin and urobilinogen. There are single test strips, e.g. diastix for glucose and multistix for multiple tests.

Automated Urinalysis

Fully automated urine analyser provided with automatic functions are presently available, e.g. Uriplus.

MICROSCOPIC EXAMINATION

Microscopic examination of urinary deposit or sediment is an essential part of urine examination. The deposits are divided into two main groups; (1) organized deposits and (2) unorganized deposits. Normal reference range for cells and casts is shown in Table 50.14.

Table 50.14: Normal reference range for cells and casts

Sediment constituents	*Normal*
Red blood cells	0-2/hpf*
White blood cells	0-5/hpf
Epithelial cells	
• Renal epithelial cells	few/hpf
• Transitional epithelial cells	few/hpf
• Squamous epithelial cells	few/lpf**
Hyaline casts	0-2/lpf
*hpf = high power field **lpf = low power field	

Procedure

- Centrifuge 10 mL of urine in a graduated conical centrifuge tube at a speed of 1500-2000 RPM for 5 minutes. The supernatant is poured off.
- Resuspend the sediment in 1mL of urine.

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- Take a small drop of the suspended urine on a glass slide.
- Mount with a coverslip and examine under the microscope.

Organized Deposit

Organized deposit consists of: (1) **cells** [blood cells (red cells and white cells) epithelial cells (renal, transitional, and squamous)], (2) **casts** (with or without inclusions), (3) **crystals,** and (4) **other** abnormal cells or formed elements.

Cells: They are expressed as number of cells per low power or high power field.

- ***Red blood cells (RBCs)***
  - Presence of RBCs (more than 2/hpf) in the urine indicates bleeding at any point in the urinary system from the glomerulus to the urethra.
  - In glomerular diseases, the urine show red cells with cellular protrusions or fragmentation and are named as **dysmorphic** (distorted morphology) red blood cells.

  Causes of hematuria (Table 50.12).
- ***White blood cells (WBCs)***
  - Increased number of WBCs (mainly neutrophils more than 5/hpf) in urine is known as **pyuria**. It is indicative of **urinary tract infection.** The causative organism of infection may be identified by bacteriological examination.
  - When accompanied by **leukocyte casts** or mixed leukocyte–epithelial cell casts, increased urinary leukocytes are considered to be of **renal origin.**

  Causes of pus cells in urine (pyuria)
  - Pyelonephritis
  - Urethritis
  - Cystitis
  - Urinary tract infection (UTI).

  Glitter cell is a swollen neutrophil with its cytoplasmic granules showing constant Brownian motion. This results in a shining or glittering appearance of the cell.

  Causes
  - Dilute or hypotonic urine
  - Chronic pyelonephritis
  - Lower urinary tract infections
- ***Epithelial cells***

  These are derived from the urinary tract (transitional and renal) or genital tract (squamous). A few (0-2/hpf) transitional cells from the bladder may be present in the normal urine and squamous cells from the vulva and vagina usually contaminate a routine specimen from women.

Casts: They are one of the organized elements which are **formed only in the kidney** and are **indicative of a renal disease.** They are formed by solidification of **Tamm Horsfall protein**, a glycoprotein secreted in the distal convoluted tubules and collecting tubules. These proteins form a fibrillar meshwork (basic matrix) and can trap any elements including cells, cell fragments or granular material (Fig. 50.9).

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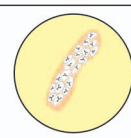
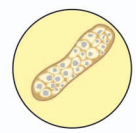
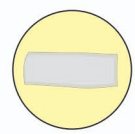
CAST	DESCRIPTION	APPEARANCE	CONDITION
Hyaline casts	• Transparent and cylindrical		• Acute glomerulonephritis (AGN) • Chronic glomerulonephritis (CGN) • Fever • Congestive heart failure
Waxy casts	• Opaque compared to hyaline casts		• End stage kidney • Chronic renal failure • Amyloidosis
Granular casts	• Cylindrical with fine or coarse granuels • Granuels derived from protein aggregates/degenerating RBCs, WBCs or tubular cells		• Tubular damage • CGN • Renal failure
Red blood cell (RBC) casts	• Hyaline or granular casts with adherent RBCs		• AGN • Lupus nephritis • Goodpasture syndrome • Subacute bacterial endocarditis • Renal infarction
Leukocyte (WBC) casts	• Hyaline or granular casts with adherent white blood cells		• Acute pyelonephritis • AGN
Epithelial casts	• Hyaline or granular casts with adherent cells from the renal tubules		• Acute tubular necrosis • Viral disease (e.g. Cytomegalovirus disease) • Drugs
Fatty casts	• Cast matrix admixed with fat droplets		• Nephrotic syndrome
Broad casts	• Diameter 2-6 times that of normal casts • Indicates tubular dilatation and/or stasis in the distal collecting duct		• Chronic renal failure (poor prognosis)
Telescoped sediment	• Displays all kinds of cells and casts		• Lupus nephritis • Hypersensitivity reaction • Subacute bacterial endocarditis

Fig. 50.9: Casts in urine and their significance

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Casts are cylindrical structures with **parallel sides** and rounded ends. They vary in size, shape and appearance. **Cylindroids** probably represent abortive casts. They have one tapering end while the other end is round and the sides are not parallel.

The casts may have only proteins (hyaline and waxy casts) or have trapped granular debris (granular casts), epithelial cells (epithelial casts), leukocytes (leukocyte casts), red blood cells (RBC casts) or fat droplets (fatty casts).

Crystals: These are not usually present in urine. Crystals of oxalates, urates and cystine are present in patients with history of renal stone, while urates alone are present in *gout.* Oxalates, urates and cystine are present in acidic urine while phosphates, calcium carbonates and ammonium urates are present in alkaline urine.

- **Crystals in acidic urine (Fig. 50.10).**

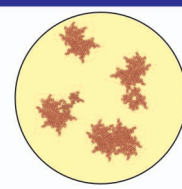
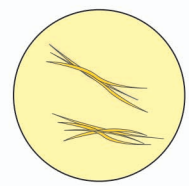
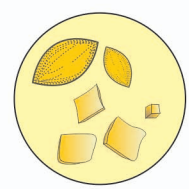
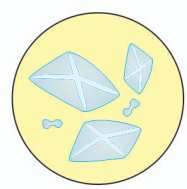
CRYSTAL	DESCRIPTION	DIAGRAM	CONDITION
Amorphous urates	• Light red amorphous sediment (referred to as "brick dust")		• Normal acidic urine • Concentrated urine as in fever and dehydration
Crystalline urates	• Needle shaped and pigmented		• Normal acidic urine
Crystalline uric acid	• Yellowish red plates, sheaves, hexagons and rhomboid shapes		• Normal acidic urine • Postchemotherapy for lymphoma and leukemia
Calcium oxalate	• Colorless, refractile and appear as envelope-shaped/dumbbell-shaped crystals		• Normal acidic urine • Nephrolithiasis • Ethylene glycol poisoning

Fig. 50.10: Crystals in acidic urine and their significance

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- **Crystals found in alkaline urine (Fig. 50.11).**

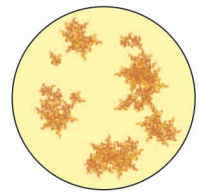
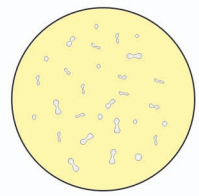
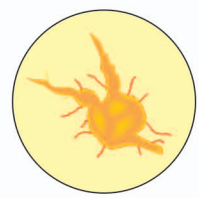
CRYSTAL	DESCRIPTION	DIAGRAM	CONDITION
Amorphous phosphates	• Colorless amorphous		• Normal alkaline urine
Crystalline phosphates—triple phosphates	• Colorless prism-shaped crystals of "coffin-lid", "knife-rest", "fern-leaf" or "star" appearance		• Normal alkaline urine • Struvite stones due to urease positive bacteria • Urinary tract infection
Calcium carbonate	• Small and colorless with dumbbell or spherical shapes		• Normal alkaline urine
Ammonium biurate crystals	• Yellow thorn apple appearance		• Normal alkaline urine

Fig. 50.11: Crystals in alkaline urine and their significance

- **Crystals found in abnormal urine (Fig. 50.12).**

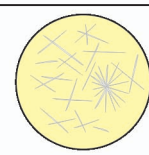
CRYSTAL	DESCRIPTION	DIAGRAM	CONDITION
Cystine	• Colorless refractile hexagonal plates with unequal sides		• Cystinuria
Tyrosine	• Fine needles in sheaves or rosettes		• Tyrosinuria

Fig. 50.12: Crystals in abnormal urine and their significance

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Others: Abnormal cells and other formed elements

- **Malignant cells:** Malignant cells exfoliated may be found in malignancies of kidney and bladder.
- **Fungi:** Yeasts (commonly *Candida* species) are seen in immunosuppressed patients, like diabetics and patients on cytotoxic drugs.
- **Parasites:** Parasites that may be found in urine are *Trichomonas, Entamoeba histolytica*, ova of *Schistosoma hematobium* and microfilaria.
- **Spermatozoa**: Sometimes sperms may be normally present and are easily recognized by their structure and motility.

Unorganized Sediments

Unorganized sediments consist of crystalline or amorphous material, the nature of which varies according to pH of the urine (acid or alkaline). Majority of these have little diagnostic or prognostic significance.

Urinary abnormalities found in few of the renal diseases are shown in Table 50.15.

Table 50.15: Urinary diseases and abnormalities observed

Disease	*Macroscopic urinalysis*	*Microscopic urinalysis*
Acute glomerulonephritis	• Gross hematuria—smoky (coffee colored) • Proteinuria—turbidity	• Neutrophils, RBCs • RBC and hyaline casts
Chronic glomerulonephritis	• Hematuria • Proteinuria	• RBCs and waxy casts ▪ Hyaline, granular
Acute pyelonephritis	• Turbid • Occasional odor	• Numerous neutrophils (many in clumps) • Leukocyte casts
Chronic pyelonephritis	• Occasional proteinuria	• Leukocytes • Type of casts depends on the extent of renal damage
Nephrotic syndrome	• Proteinuria • Fat droplets	• Fatty casts and oval fat bodies (fat-filled renal tubular epithelial cells)

SUMMARY

- Urine analysis reflects the state of the function of kidneys and urinary tract as well as provide information about nonrenal disorders.
- Urinalysis consists of physical, chemical and microscopic examination.
- In physical examination the volume, color, transparency, odor, reaction and specific gravity are noted.
- Chemical examination is done to detect proteins, reducing substances (like glucose), ketone bodies, bilirubin, urobilinogen, bile salts and blood.
- Presently the chemical methods are replaced by multistix. These are simple, sensitive and rapid. Fully automated urine analyser is presently available.

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- Microscopic examination of urine sediment is an essential part of urine examination. These sediments are divided mainly into two groups namely organized and unorganized deposits.
- Organized deposit consists of cells (RBCs, WBCs and epithelial cells), casts, crystals, abnormal cells and other formed elements.
- Unorganized sediments consist of crystalline or amorphous material, majority of which have little diagnostic or prognostic significance.

SELF-ASSESSMENT EXERCISES

I. Short Notes

1. Physical examination of urine
2. Volume of urine
3. Routine detailed examination of urine
4. Causes of increased and decreased specific gravity
5. What is fixed specific gravity of urine? What is its importance?
6. Causes of massive proteinuria and tests for proteinuria
7. Chyluria
8. Albuminometer
9. Causes and tests for glycosuria
10. Principle, method and interpretation of Benedict's qualitative test for sugar
11. Causes and tests for ketonuria
12. Causes of nondiabetic ketonuria
13. Rothera's test
14. Urine urobilinogen in jaundice
15. Tests for blood in urine
16. List the causes of hematuria/renal causes of hematuria
17. Microscopic examination of urine
18. Urinary casts/sediments
19. Enumerate the urinary casts
20. Urinary crystals

II. Multiple Choice Questions

1. **The cola colored urine is seen with**
   A. Intravascular hemolysis
   B. Alkaptonuria
   C. Extravascular hemolysis
   D. Acute pyelonephritis
2. **Chyluria is NOT found in**
   A. Filariasis
   B. Malaria
   C. Abdominal lymphadenopathy
   D. Abdominal tumors

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3. **Fixed specific gravity of 1.010 is found in**
   A. Chronic renal failure
   B. Acute glomerulonephritis
   C. Nephrotic syndrome
   D. Renal cell carcinoma
4. **The cause for massive proteinuria is**
   A. Nephrotic syndrome
   B. Nephritic syndrome
   C. Acute renal failure
   D. Adult polycystic kidney
5. **Increased urobilinogen in urine is NOT a feature of**
   A. Thalassemia
   B. Sickle cell anemia
   C. Hereditary spherocytosis
   D. Obstructive jaundice
6. **Myoglobinuria is seen in**
   A. Crush injury to muscle
   B. Abdominal surgery
   C. Trauma to the kidney
   D. Massive hemorrhage

ANSWERS

1. A **2.** B **3.** A **4.** A **5.** D **6.** A

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CHAPTER 51

Body Fluids

CHAPTER OUTLINE

- ❖ Introduction
- ❖ Examination of Body Fluids
- ❖ Examination of Synovial Fluids

INTRODUCTION

Body fluids are lubricating fluids present within the body cavities. Normally, a small amount of fluid is present within the body cavities which keeps the surfaces moist and lubricated so that the movement of the adjacent or the opposing membrane surfaces occurs with minimal friction. Increase in the volume of the fluid in these cavities is known as **effusion**. The commonly examined body fluids in the laboratory include **pleural, pericardial, peritoneal and synovial fluid.**

Effusions may be broadly divided into **transudate** and **exudate**; the differences between them are shown in Table 51.1.

Table 51.1: Differences between transudate and exudate

Characteristics	*Transudate*	*Exudate*
Mechanism	Filtrate of plasma, due to non-inflammatory process (increased hydrostatic pressure) with normal vascular permeability	Increased vascular permeability due to inflammatory process
Appearance	Clear, serous	Cloudy/purulent/hemorrhagic/chylous
Color	Straw yellow	Yellow to red
Specific gravity	<1.018	>1.018
Protein	Low, <2 gm/dL, mainly albumin	High, >2 gm/dL
Clot	Absent	Clots spontaneously because of high fibrinogen
Cell count	Low count	High count
Type of cells	Few lymphocytes and mesothelial cells	Increased neutrophils in acute and lymphocytes in chronic inflammation
Bacteria	Absent	Usually present
LDH	Low	High

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Specimen Collection

The body fluid is collected in a clean, dry container under aseptic precautions and atraumatically to avoid mixing with fresh blood. The fluid is collected in the following three sterile test tubes:

- Chemical examination: Fluoride tube
- Microscopic examination: EDTA tube
- Bacteriological examination: Plain tube (without anticoagulant).

They should be examined as early as possible to prevent chemical changes, growth of bacteria and disintegration of cells.

EXAMINATION OF BODY FLUIDS

Physical Examination

Note volume, color and appearance.

Color: Pleural, pericardial and ascitic (peritoneal) fluids are usually clear and straw colored.

- Uniform **blood** stained fluid suggests malignancy involving the organs/tissues surrounding the respective body cavity.
- **Turbid** fluid may be due to high cell count or high protein content.
- **Chylou**s with milky appearance usually indicates high lipid content due to lymphatic obstruction.

Transudate VS. exudate: It is important to differentiate whether the fluid is a transudate or exudate (Table 51.1).

- **Transudate** is usually seen in all body cavities with diseases like heart failure and hypoalbuminemic conditions (e.g. nephrotic syndrome). Cirrhosis results in prominent ascites, but may also cause pleural effusion.
- Exudate usually suggests infection or malignancy.

Chemical Examination

- Protein estimation: This helps to differentiate transudate from exudate (Table 51.1).
- Glucose estimation: Low glucose in the body fluids usually suggests bacterial infection (including tuberculosis), malignancy or nonspecific inflammation.
- Measurement of amylase in ascitic fluid in patients with pancreatic lesions.

Microscopic Examination

- ***Cell count*** is done similar to total WBC count using improved Neubauer chamber.
  - Normal: Few mesothelial cells (lining cells of body cavities) and lymphocytes are seen.
- ***Differential WBC count***

  Procedure: Centrifuge the body fluid and from the sediment prepare the smears (at least 2).

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Stains:

- Leishman's stain: Stain one smear with Leishman's stain and count 100 cells and express the differential count.
- Gram's stain/acid fast stain: These stains are useful in suspected cases of infective/tubercular infections.

- *Cytological examination for malignant cells*
  - **Procedure:** The body fluid is centrifuged; smears are made from the sediment and fixed immediately in absolute alcohol. The smears are stained by Papanicolaou stain (refer Chapter 56). Hematoxylin and eosin stain or Giemsa may also be used.
  - Cytospin is a better alternative for making smears.

Microbiological Examination

Culture is done to identify the organism in cases of effusion due to infections.

EXAMINATION OF SYNOVIAL FLUIDS

Uses: Examination of synovial fluid is useful in the diagnosis of joint disorders.

- Infective arthritis (septic arthritis, rheumatic)
- Gouty arthritis (metabolic disorder)
- Rheumatoid arthritis (autoimmune disorder)
- Degenerative arthritis.

Laboratory examination: It consists of (1) physical examination,(2) microscopic examination, (3) chemical examination, and (4) microbiological examination.

Physical Examination

a. Color and appearance (Table 51.2)

Table 51.2: Color and appearance of synovial fluid

Appearance	*Condition*
Normal	Clear, straw colored and viscous. It does not clot
Turbid	Infection and inflammation of the joint space, due to the presence of crystals, amyloid and cartilage fragments
Purulent	Septic arthritis
Red or brown supernatant	Hemarthrosis or in a traumatic tap

b. Viscosity test

Synovial fluid is viscous due to the presence of hyaluronic acid. The viscosity of the synovial fluid decreases in inflammatory joint disorders due to the breakdown of hyaluronic acid by the enzyme hyaluronidase.

c. Mucin clot test: Hyaluronic acid forms a compact clot when mixed with the acetic acid. Low concentration of hyaluronic acid does not allow the formation of a firm clot.

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Add few drops of synovial fluid to 20 mL of 5% acetic acid in a small beaker. A good clot is formed if the synovial fluid is normal.

- In inflammatory diseases of the joint, there is poor clot formation due to degrading enzymes from the inflammatory cells (e.g. tuberculous arthritis).
- Noninflammatory joint disorders show good clot formation whereas hemorrhagic synovial fluid prevents clot formation due to dilution of fluid.
- Fair to poorly formed clot is seen in rheumatoid arthritis, gout and pseudogout.

Microscopic Examination

- The **total leukocyte count** is estimated similar to total WBC count using improved Neubauer chamber.
- **Differential leukocyte count**
  - If polymorphs are more than 70%, it indicates bacterial arthritis.
  - Noninflammatory arthropathies (osteoarthritis) are associated with lymphocytes and macrophages.
- **Wet smear examination**
  Centrifuge the synovial fluid and take the sediment on a glass slide and cover it with a coverslip. Observe the slide first under low power objective, then under high power objective with reduced light and carefully note for the presence of following crystals:
  - Urate crystals are needle shaped, highly birefringent and are seen in gouty arthritis.
  - Rhomboid calcium pyrophosphate crystals are seen in pseudo-gout.
  - Cholesterol crystals are seen in rheumatoid arthritis.
  - Crystals can be confirmed using polarized microscopy.

Chemical Examination

- Glucose estimation: Significance is similar to glucose in the body fluids.
- Protein estimation: Significance is similar to proteins in the body fluids.

Microbiological Examination

Synovial fluid culture is recommended in suspected cases of pyogenic/tubercular arthritis.

SUMMARY

- Body fluids are normally present in small amounts within body cavities. Increase in the volume of the fluid in these cavities is known as effusion.
- Commonly examined body fluids include pleural, pericardial, peritoneal and synovial fluid. It is important to differentiate whether the fluid is a transudate or an exudate.
- Examination of body fluid consists of physical, microscopic, chemical and microbiological examination. In physical examination volume, color and appearance are noted.

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Microscopically, fluids are examined for total number of cells, types of cells and malignant cells, if any. Chemical examination consists of estimation of glucose, protein and other specific tests depending on the type of body fluid. Culture of the fluid is done in cases with inflammatory or infective disorders.

- In synovial fluid, wet smear examination can be done to identify the crystals.

SELF-ASSESSMENT EXERCISES

I. Short Notes

1. Differences between exudate and transudate
2. Synovial fluid examination
3. Examination of body fluids

II. Multiple Choice Questions

1. **All the following are features of transudate EXCEPT**
   A. Specific gravity less than 1.018
   B. Proteins less than 2 gm/dL
   C. Low cell count
   D. High LDH
2. **Viscosity test and mucin clot tests are done on**
   A. Pericardial fluid
   B. Pleural fluid
   C. Ascitic fluid
   D. Synovial fluid

ANSWERS

1. D **2.** D

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CHAPTER 52

Cerebrospinal Fluid Examination

CHAPTER OUTLINE

- ❖ Introduction
- ❖ Examination of CSF

INTRODUCTION

Cerebrospinal fluid (CSF) is formed within the ventricles and circulates in the subarachnoid space (between arachnoid and pia matter) and in the ventricles. The total volume of CSF in adults ranges from 90 to 150 mL. It is a medium for transfer of substances from brain and spinal cord into the blood.

Importance of CSF examination: Analysis of the CSF is of diagnostic importance in conditions like meningitis or primary/metastatic tumor of CNS with CSF involvement.

Collection of CSF: CSF is usually obtained by lumbar puncture (LP) using an LP needle under strict aseptic conditions.

- ***Lumbar puncture needle*** **(Fig. 52.1):** The needle measures 10-12 cm in length. In children, a shorter needle is used. It has a needle and a stilette. The stilette has a pin which fits into the slot of the head of the needle and helps to keep the needle patent.

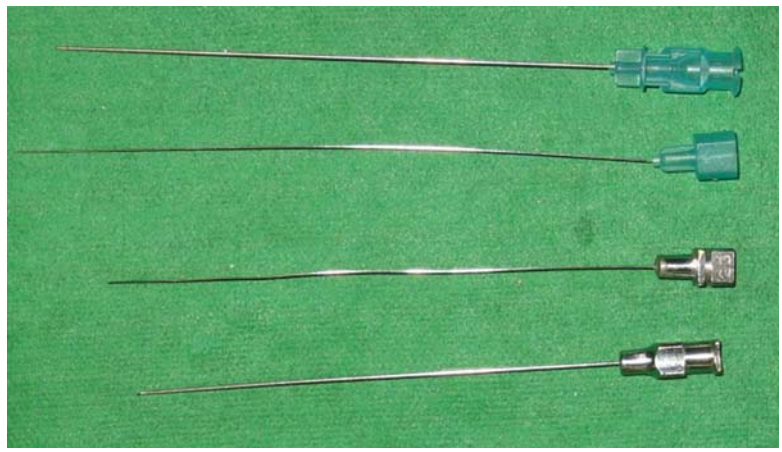


Fig. 52.1: Lumbar puncture needles with stilette (disposable needle with green plastic hub above)

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Lumbar puncture: In **adults**, CSF is normally collected in the midline of the lower back in the **3rd lumbar space** and in children in the 4th lumbar space.
Cisternal puncture: It is done in spinal cord block, vertebral deformity or infections in the tissues where lumbar puncture is usually done.
Ventricular puncture: It is performed in infants who have open fontanella.

Method of Collection

Normally CSF is collected in three sterile test tubes. The amount of CSF collected should not exceed 6 to 8 mL.

- Microbiological examination and culture: The first few drops of CSF are collected for culture in the first tube.
- Biochemical tests and immunological studies: Collect about 2.5 mL of CSF in the second tube for biochemical tests.
- Cell count, differential count and cytological studies: Collect about 2.5 mL of CSF in the third tube for cell count and cytological examination.

Note:

- CSF should be examined immediately within 1 hour.
- CSF should not be refrigerated, especially if the sample is meant for culture, because the most common causative agent for meningitis (Neisseria) is destroyed when exposed to cold temperature.

Indications for Lumbar Puncture

A. Diagnostic Indications

- Infection
  - Meningeal infection:
    - Bacterial
      - Pyogenic
      - Tuberculosis
      - Syphilitic: To differentiate general paresis of insane, tabes dorsalis and meningeal syphilis.
    - Viral
    - Fungal
  - Encephalitis
- Subarachnoid hemorrhage
- Primary or metastatic malignancy (e.g. acute leukemia, lymphoma)
- Demyelinating diseases: Multiple sclerosis and subacute sclerosing panencephalitis (SSPE), Guillain-Barré syndrome

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- Spinal canal blockage leading to elevated intracranial tension (spinal cord tumors)
- Injecting the radio-opaque dye for myelography.

B. Therapeutic Indications

- Spinal anesthesia
- Intrathecal injection of chemotherapeutic drugs for CNS prophylaxis/relapse of ALL, lymphomas.

Contraindications for Lumbar Puncture

- Raised intracranial pressure
- Local infective lesion
- Disseminated sclerosis
- Brain tumor.

Complications of Lumbar Puncture

- Herniation of cerebellum through the foramen magnum due to raised intracranial pressure.
- Hematoma, either extradural or subdural.
- Introduction of infection by the LP needle through the infected skin or subcutaneous tissue.

EXAMINATION OF CSF

Physical Examination

CSF pressure: The normal CSF pressure in adults is 90-180 mm of water in the lateral position, while in infants and children it ranges from 40-100 mm of water reaching the adult level by 6 to 8 years.

- ***Causes of raised CSF pressure:*** Meningitis, cerebral edema, and mass lesions in brain.
- ***Causes of decreased CSF pressure:*** Dehydration and circulatory collapse.

Color and Appearance: Normal CSF is clear and watery.

- Turbidity of CSF is due to pus or RBCs.
- Xanthochromia (yellow CSF) results from:
  - Old hemorrhage
  - Obstructive jaundice
  - Excess of protein
  - Froin syndrome.

Differentiation of subarachnoid hemorrhage from a traumatic puncture

- The presence of blood in CSF may be due to trauma during lumbar puncture or subarachnoid hemorrhage.
- In traumatic tap, first few drops are hemorrhagic and subsequent ones are clear, while in subarachnoid hemorrhage blood is uniformly mixed with CSF.

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- After centrifugation, the supernatant fluid is clear with a traumatic tap, whereas the supernatant appears xanthochromic (a faint pink, orange or yellow color caused by release of hemoglobin from hemolyzed red blood cells) in subarachnoid hemorrhage.

- **Clot formation:** Normal CSF does not clot. When blood-brain barrier is disturbed, fibrinogen appears in CSF. Fibrinogen gets converted to fibrin and forms clot.

  Causes of fibrin clot:
  - Meningitis
    - In tuberculous meningitis, the clot is fine, delicate and typically described as cob-web appearance.
    - In purulent meningitis, large clot is formed.
  - Tumors of CNS
  - Polyneuritis.

Microscopic Examination

Total cell count: Normal CSF usually contains no cells, although cell count of 0-5 lymphocytes/μL is considered as normal**.** An increased cell count is known as **pleocytosis.** Total cell count should be done immediately on undiluted CSF, since after a few hours, pus cells stick to each other and to the sides of the tube or degenerate. Cell counts are performed in a manual counting chamber, either in a Fuchs–Rosenthal or improved Neubauer chamber.

Significance

- Neutrophils are increased in acute pyogenic meningitis.
- Lymphocytes are increased in viral, syphilitic, tuberculous and fungal meningitis.

Increased cell count should always be confirmed by bacterial or serological tests.
India-ink preparation is used for diagnosis of cryptococcal meningitis.
In tuberculous meningitis, acid fast stain can detect tuberculous bacilli.

Biochemical Evaluation

- Proteins are elevated in meningitis and glucose level is reduced due to utilization by the microbes. These changes are more marked in pyogenic meningitis.
- Chloride reduction in tuberculous meningitis is due to general chloride deficiency because of dehydration rather than any specific effect of mycobacteria.

Table 52.1: CSF findings in meningitis

	Normal	*Pyogenic*	*Tuberculous*
Physical examination	Clear and colorless	Turbid and forms coagulum	Clear and colorless, forms cobweb on standing due to coagulation of increased fibrinogen
Total protein	20-40 mg/100 mL	50-200 mg/100 mL	50-150 mg/100 mL
Glucose	45-80 mg/100 mL	0-20 mg/100 mL	20-50 mg/100 mL
Chlorides	720-750 mg/100 mL	600-700 mg/100 mL	450-600 mg/100 mL

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Cells			
Polymorphs	Usually absent	150-2000/µL	0-5 cells/µL
Lymphocytes	0-5 cells/µL	5-50 cells/µL	500-700 cells/µL

µL = microlitre

SUMMARY

- CSF analysis is of diagnostic importance in conditions like meningitis or primary/metastatic tumor of CNS with CSF involvement.
- CSF is obtained by lumbar puncture using an LP needle under strict aseptic conditions.
- Lumbar puncture has both diagnostic and therapeutic indications.
- Examination of CSF consists of physical, microscopic and biochemical analysis.
- Microscopically, neutrophils are increased in acute pyogenic meningitis and lymphocytes are increased in viral and tuberculous meningitis.

SELF-ASSESSMENT EXERCISES

I. Short Notes

1. Indications for lumbar puncture
2. CSF findings in tuberculous meningitis
3. CSF findings in pyogenic meningitis
4. CSF findings in viral meningitis

II. Multiple Choice Questions

1. **CSF forms cobweb on standing in**
   A. Tuberculous meningitis
   B. Fungal meningitis
   C. Pyogenic meningitis
   D. Viral meningitis
2. **One of the indication for lumbar puncture is**
   A. Raised intracranial pressure
   B. Disseminated sclerosis
   C. Brain tumor
   D. Subarachnoid hemorrhage

ANSWERS

1. A **2.** D

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CHAPTER 53

Semen Analysis

CHAPTER OUTLINE

- ❖ Introduction
- ❖ Different Procedures of Semen Analysis
- ❖ Cryopreservation of Spermatozoa

INTRODUCTION

Semen (seminal fluid) consists of spermatozoa (sperms) and the fluid part. About 40% cases of infertility are due to abnormalities in semen and therefore semen analysis is the first test to be performed while investigating for infertility. **Defect** of sperms may be **quantitative** (absence of sperms, lack of enough sperms) or a **qualitative**.

Indications for Semen Analysis

- Assessment of fertility/**infertility.**
- Determine the **effectiveness of vasectomy.**
- Determine the **suitability** of semen **for artificial insemination**.
- **Medicolegal purpose**: In alleged rape cases, vaginal pool smears are examined to detect sperms.

Collection of the Sample

Patient is asked to collect the semen by masturbation after a minimum of 2 days and a maximum of 7 days of sexual abstinence. Specimen should be collected in a clean, dry, wide mouthed plastic/ glass container. Collection of condom sample is not advisable because they often contain spermicidal agents which impair the sperm motility.

DIFFERENT PROCEDURES OF SEMEN ANALYSIS

They can be mainly divided into three types: standard, optional and research procedures.

Standard Procedures

Physical Examination

- ***Liquefaction:*** Immediately after ejaculation, the semen is normally a semisolid coagulated mass. At room temperature, the semen usually begins to liquefy (become thinner) within a

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few minutes and completely liquefies within 15 minutes. Within 30 minutes it becomes more homogeneous and watery.

- ***Semen viscosity:*** Fresh semen is fairly viscid and the viscosity can be estimated by gently aspirating semen into a wide-bore (approximately 1.5 mm diameter) plastic disposable pipette, allowing the semen to drop by gravity. Normal semen falls drop by drop and if viscosity is abnormal, the drop will form a thread more than 2 cm long. Normal viscosity is important, since increase in viscosity affects sperm motility**.**
- ***Appearance*****:** Freshly ejaculated semen is an opaque, white gray and viscid fluid. After liquefaction it has homogeneous grey-opalescent appearance. Semen may have red-brown color when red blood cells are present (hemospermia) or yellow in patients with jaundice or ingestion of certain vitamins or drugs.
- ***Semen volume:*** Normal volume ranges from 1.4 to 1.7 mL per ejaculate. Low semen volume may be due to obstruction of the ejaculatory duct, congenital bilateral absence of the vas deferens or can also be due to difficulty in collection. High semen volume may be due to active exudation in cases of inflammatory lesions of the accessory organs.
- ***Semen pH:*** Alkaline and ranges from 7.2 to 8 (≥7.2). The pH should be measured after liquefaction, preferably after 30 minutes.

Microscopic Examination

- ***Sperm aggregation or agglutination***
  - **Aggregation of spermatozoa:** The adherence either of immotile spermatozoa to each other or of motile spermatozoa to mucus strands, nonsperm cells or debris is considered to be nonspecific aggregation and should be noted.
  - **Agglutination of spermatozoa:** Agglutination refers to **motile spermatozoa sticking to each other,** head-to-head, tail-to-tail or in a mixed way. Any motile spermatozoa that stick to each other by their heads, tails or midpieces should be recorded. The grading of agglutination of spermatozoa is shown in Table 53.1.

Table 53.1: Grading of agglutination of spermatozoa

Grade	*Pattern of agglutination*
Grade 1:Isolated	<10 spermatozoa per agglutinate and many free spermatozoa
Grade 2:Moderate	10–50 spermatozoa per agglutinate and free spermatozoa
Grade 3:Large	Agglutinates of >50 spermatozoa and some spermatozoa still free
Grade 4:Gross	All spermatozoa agglutinated and agglutinates interconnected

- ***Cellular elements other than spermatozoa:*** During microscopic examination search should be made for the presence of cells other than spermatozoa. Some of these cells may be clinically relevant, which includes epithelial cells from the genitourinary tract and "round cells" (leukocytes and immature germ cells).
- ***Assessment of sperm motility:*** Motility of the sperms helps in penetration of cervical mucus and migration of the sperms into the fallopian tube. In normal semen, 38 to 42% of sperms should be motile (progressively motile and non-progressively motile). This is assessed by placing a drop of liquefied semen on a clean glass slide with a coverslip placed over it and

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examining under microscope. According to the WHO (2010), the motility of each spermatozoon (plural is spermatozoa) is categorized as progressively motile (PR), non-progressively motile (NP) and immotile (IM). This grading system (Table 53.2) has replaced the older grading system of a, b, c or d (I to IV) grades.

Table 53.2: WHO (2010) system of grading sperm motility

Type of motility	*Characteristic features*
Progressive motility (PR)	Spermatozoa moving actively, either linearly or in a large circle, regardless of speed
Non-progressive motility (NP)	All other patterns of motility with an absence of progression, e.g. swimming in small circles, the flagellar force hardly displacing the head, or when only a flagellar beat can be observed
Immotility (IM)	No movement

- ***Sperm vitality:*** It is important to know whether immotile spermatozoa are alive or dead. Normally 55 to 63 % live forms are observed. Vitality of the spermatozoa is estimated by identifying those with an intact cell membrane and is especially important for samples with less than about 40% progressively motile spermatozoa. The percentage of live spermatozoa is assessed either by dye exclusion or by hypotonic swelling.
  - **Dye exclusion method:** It is based on the principle that damaged plasma membranes, such as thoses found in non-vital (dead) cells, allow entry of membrane-impermeant stains.
    - *Vitality test using eosin–nigrosin:* The nigrosin helps to increases the contrast between the background and the sperm heads, thereby makes them easier to identify. The dead sperms are stained by eosin.
    - *Vitality test using eosin alone:* This is a simple and rapid method, but the wet preparations cannot be stored for quality control purposes.
  - **Hypo-osmotic swelling test:** The principle is based on the fact that only cells with intact membranes (live cells) will swell in hypotonic solutions.
- ***Total sperm count***

  Sperm count is carried out in an improved Neubauer chamber using a Thoma pipette in a dilution of 1 in 20 (as for total leukocyte count). The composition of semen diluting fluid is shown in Table 53.3

Table 53.3: Composition of semen diluting fluid

Constituents	*Quantity*
Sodium bicarbonate	5 g
Formaline	1 mL
Distilled water	100 mL

Normal range of sperm count: 33-46 millions/ejaculate.

- ***Sperm morphology*** **(Fig. 53.1):** Smear prepared from semen is fixed and stained with Papanicolaou stain (Chapter 56) to identify the morphological features.

  Normal: Spermatozoa have a head, neck, middle piece (midpiece), principal piece and endpiece. Since the endpiece is difficult to see under light microscope, the spermatozoa can

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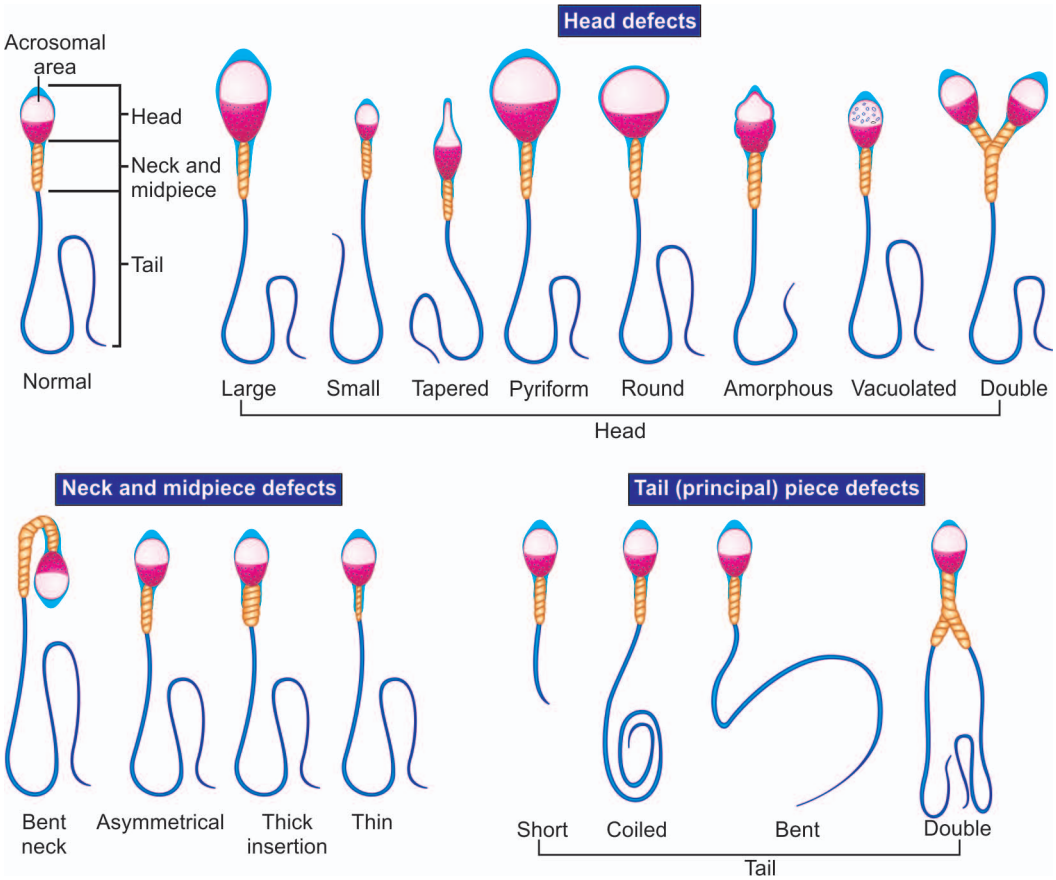


Fig.53.1: Sperm morphology of both normal and abnormal forms

be considered to consists of a head (and neck) and tail (midpiece and principal piece). Normally more than 30% of sperms have normal morphology. For a spermatozoon to be considered as normal, both its head and tail must be morphologically normal. All other forms should be considered as abnormal.

- **Head:** It is oval in shape with smooth outline. It has an acrosomal region which constitutes 40–70% of the head area. The acrosomal region does not contain large vacuoles and not more than two small vacuoles which do not occupy more than 20% of the sperm head. The post-acrosomal region of the head does not contain any vacuoles.
- **Neck and midpiece:** It is slender, regular and has the same length as the sperm head. The major axis of the midpiece is in line with the major axis of the sperm head.
- **Tail (principal) piece**: It has a uniform caliber and is thinner than the midpiece. It is about 10 times the head length and may be looped back on itself.

Classification of abnormal sperm morphology (Fig. 53.1): Abnormal spermatozoa usually have a lower fertilizing capacity. The abnormalities in morphology can be classified as;

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- **Head defects:**
  - Abnormal size: Large or small head
  - Abnormal shape: Pointed/tapered, round, pyriform, amorphous
  - Acrosomal defects
    - Vacuolated acrosomal region: More than two vacuoles or >20% of the head area occupied by vacuoles. Presence of vacuoles in the post-acrosomal region.
    - Abnormality of acrosomal areas: Small or large acrosomal areas (<40% or >70% of the head area)
  - Abnormal number: Double-headed
- **Neck and midpiece defects:**
  - Sharply bent neck
  - Asymmetrical insertion of the midpiece into the head
  - Thick or thin or irregular midpiece
- **Tail (principal) piece defects:** These abnormalities include short or bent or coiled or double tail.

Tests for Antibody Coating of Spermatozoa

The agglutination of spermatozoa may be the result of the anti-sperm antibodies (ASAs) or other factors present in the semen. ASAs in the semen belong to IgA and IgG types and it may also be found in sperm-free fluids (seminal plasma, blood serum and cervical mucus).

- ***Tests for antibodies on spermatozoa ("direct tests"):*** Two direct tests include the mixed antiglobulin reaction (MAR) test and the immunobead (IB) test.
- ***Tests for anti-sperm antibodies in sperm-free fluids ("indirect" tests)***.

Optional Procedures

These tests are not necessary for routine semen analysis, but may be useful in certain circumstances.

Indices of Multiple Sperm Defects

Spermatozoa which morphologically appear abnormal often have multiple defects (of the head, midpiece or principal piece, or combinations of these defects). A detailed examination of the incidence of morphological abnormalities is more useful than a simple evaluation of the percentage of morphologically normal spermatozoa. Three useful indices of multiple sperm defects are:

- ***Multiple anomalies index (MAI):*** It is the mean number of anomalies per abnormal spermatozoon. It includes all the head, midpiece and principal piece anomalies.
- ***Teratozoospermia index (TZI):*** In this, a maximum of four defects (one from each component of spermatozoon such as midpiece, tail piece) counted, irrespective of real number of anomalies in the abnormal spermatozoon.
- ***Sperm deformity index (SDI):*** It is the number of defects divided by the total number of spermatozoa.

These indices have been correlated with fertility *in vivo* (MAI and TZI) an *in vitro* (SDI).

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Panleukocyte (CD45) Immunocytochemical Staining

All leukocytes express a CD45 which can be detected by immunocytochemical staining. This is useful for distinguishing between leukocytes and germ cells.

Interaction Between Spermatozoa and Cervical Mucus

Cervical mucus favors sperm penetration during the mid-cycle of the menstrual cycle.

- ***In vivo (postcoital) test***: The postcoital test is used to determine the number of active spermatozoa in the cervical mucus and to evaluate sperm survival and sperm behavior few hours after coitus.
- ***In vitro tests***: Assessment of sperm–cervical mucus interaction may also be undertaken using *in vitro* penetration tests.

Biochemical Assays for Accessory Sex Organ Function

Qualitative defects in semen may be due to testicular production of abnormal spermatozoa or from post-testicular damage to spermatozoa in the epididymis or the ejaculate from abnormal accessory gland secretions. Secretions from accessory glands can be measured to assess gland function, e.g. citric acid, zinc, γ-glutamyl transpeptidase and acid phosphatase for the prostate; fructose and prostaglandins for the seminal vesicles; free L-carnitine, glycerophosphocholine (GPC) and neutral α-glucosidase for the epididymis.

- ***Measurement of zinc in seminal plasma:*** The amount of zinc in semen is a reliable measure of prostate gland secretion. The lower reference limit for zinc is 2.4 μmol per ejaculate.
- ***Measurement of fructose in seminal plasma:*** Fructose is the main sugar in the semen reflects the secretory function of the seminal vesicles. It is reduced when there is androgen deficiency or obstruction to the ejaculatory duct. There is inverse relationship between sperm count and fructose level. The lower reference limit for fructose is 13 μmol per ejaculate.
- ***Measurement of neutral α-glucosidase in seminal plasma*:** Seminal plasma contains both a neutral α-glucosidase isoenzyme, which originates in the epididymis, and an acid isoenzyme contributed by the prostate. The lower reference limit for neutral α-glucosidase is 20 mU per ejaculate.
- ***Acid phosphatase test:*** It is used in alleged rape cases and is performed on vaginal aspirates. Normal level of acid phosphatase in semen is less than or equal to 200 U/ejaculate.

Computer-aided Sperm Analysis

Computer aided sperm analysis (CASA) can be used to measure sperm concentration. By the use of fluorescent DNA stains with CASA, the concentration of motile sperm and percentage motility can be determined.

Research Procedures

Several diagnostic tests have been developed which are capable of detecting the precise nature of the processes that are defective in the spermatozoa of infertile men. These are mentioned in Table 53.4.

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Table 53.4: Different procedures of semen analysis and its reference limits/significance

Parameter	*WHO (2010) lower reference limit (range)/significance*
A. STANDARD PROCEDURES	
Physical examination	
Liquefaction	<30 minutes
Semen viscosity	Thread less than 2 cm long
Appearance: Color	Translucent, gray-white or opalescent
Semen volume	1.5 (1.4 -1.7) mL
Semen pH	≥7.2
Microscopic examination	
Sperm aggregation or agglutination	Its presence is suggestive of the presence of anti-sperm antibodies
Cellular elements other than spermatozoa which are clinically significant.	Epithelial cells from the genitourinary tract and "round cells" (leukocytes and immature germ cells)
Assessment of sperm motility	
• Total motility (PR + NP)	40 (38-42)%
• Progressive motility (PR)	32 (31-34)%
▪ *Sperm vitality (live spermatozoa)*	58 (55-63)%
Total sperm count	39 (33-46) × 10^9 per ejaculate
Sperm morphology (normal forms)	4 (3.0-4.0)%
Tests for antibody coating of spermatozoa	
Tests for antibodies on spermatozoa ("direct tests")	
• MAR test (motile spermatozoa with bound particles)	<50%
• Immunobead test (motile spermatozoa with bound beads)	<50%
Tests for anti-sperm antibodies in sperm-free fluids ("indirect" tests)	<50%
B. OPTIONAL PROCEDURES	
Indices of multiple sperm defects	
Multiple anomalies index (MAI)	1.04-2.38
Teratozoospermia index (TZI)	1.17-2.07
Sperm deformity index (SDI)	
Panleukocyte (CD45) immunocytochemical staining	<1.0 × 10^6 per mL
Interaction between spermatozoa and cervical mucus	Presence of any spermatozoa with progressive motility indicates normal cervical function
In vivo (postcoital) test	
In vitro tests	
Biochemical assays for accessory sex organ function	
Seminal zinc	≥2.4 µmol/ejaculate
Seminal fructose	≥13 µmol/ejaculate
Seminal neutral α-glucosidase	≥20 mU/ejaculate
Total acid phosphatase	≥200 U/ejaculate
Computer-aided sperm analysis	
C. RESEARCH PROCEDURES	
Reactive oxygen species	
Human sperm–oocyte interaction tests	
Human zona pellucida binding tests	
Assessment of the acrosome reaction	
Zona-free hamster oocyte penetration test	
Assessment of sperm chromatin	

PR = Progressive motility: NP = Non-progressive motility; MAR = Mixed antiglobulin reaction

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CRYOPRESERVATION OF SPERMATOZOA

One of the methods of storing **s**permatozoa is by cryopreservation.

Indications for Cryopreservation

- **Donor semen**: Donor spermatozoa can be used for artificial insemination (AI), intrauterine insemination (IUI), in-vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI).
- **Fertility preservation:** Semen may be stored before a man undergoes a procedure or exposure that might prevent or impair his fertility. These include vasectomy, treatment with cytotoxic agents or radiotherapy.
- **Infertility treatment:** Spermatozoa may be stored for artificial insemination by husband's semen.
- **Minimizing infectious disease transmission:** For men with HIV controlled by antiretroviral therapy, semen samples with an undetectable viral load may be stored for IUI, IVF or ICSI, to attempt conception which reduces the risk of HIV transmission to the female partner.

Terminologies used in semen analysis (Table 53.5).

Table 53.5: Terminologies used in semen analysis

Terminology	*Description*
Aspermia	No semen
Azoospermia	No spermatozoa in the ejaculate
Oligospermia	Total number of spermatozoa below the lower reference limit
Asthenospermia	Percentage of progressively motile (PR) spermatozoa below the lower reference limit
Normozoospermia	Total number of spermatozoa, and percentages of PR and morphologically normal spermatozoa, equal to or above the lower reference range
Teratozoospermia	Percentage of morphologically normal spermatozoa below the lower reference limit
Necrozoospermia	Low percentage of live and high percentage of immotile spermatozoa in the ejaculate
Hemospermia	Presence of RBCs in the ejaculate

The suffix "spermia" refers to the ejaculate and "zoospermia" to the spermatozoa

SUMMARY

- Semen analysis is used for assessment of fertility, effectiveness of vasectomy, to determine its suitability for artificial insemination and medicolegal purpose.
- Semen analysis is mainly divided into three types: standard, optional and research procedures.
- Standard procedure includes physical examination, microscopic examinations and tests for antibody coating spermatozoa.
- Normal sperm count ranges from 33 to 46 millions/ejaculate.
- Morphologically, spermatozoa have a head, neck, middle piece (midpiece), principal piece and end piece and abnormality may involve any one or more of these components.

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SELF-ASSESSMENT EXERCISES

I. Short Notes

1. Semen analysis
2. Semen analysis in a case of suspected infertility
3. Morphological abnormalities of sperm

II. Multiple Choice Questions

1. **WHO reference range for normal sperm count per ejaculate is more than**
   A. 10 million
   B. 20 million
   C. 30 million
   D. 40 million
2. **The amount of zinc in semen is a reliable measure of secretion of**
   A. Prostate gland
   B. Epididymis
   C. Seminal vesicle
   D. Leydig cells
3. **Fructose present in the semen is produced by**
   A. Prostate glands
   B. Spermatogonia
   C. Epididymis
   D. Seminal vesicle
4. **Neutral α-glucosidase found in semen is derived from**
   A. Leydig cells
   B. Prostate glands
   C. Epididymis
   D. Seminal vesicle

ANSWERS

1. D **2.** A **3.** D **4.** C

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CHAPTER 54

Pregnancy Test

CHAPTER OUTLINE

- ❖ Introduction
- ❖ Pregnancy Tests

INTRODUCTION

Pregnancy test detects human chorionic gonadotropin (HCG), which is a hormone secreted by the viable placental tissue. The test may be employed for either qualitative or quantitative estimation of HCG. The term pregnancy test is a misnomer, as it has other diagnostic indications also.

Indications:

- ***Pregnancy***
  - Diagnosis and confirmation of early normal uterine pregnancy.
  - Evaluation of ectopic pregnancy.
  - To monitor pregnancy following *in vitro* fertilization and embryo transfer.
- ***Diagnosis and follow up of***
  - Gestational trophoblastic disease:
    - Hydatidiform (vesicular) mole
    - Invasive mole
    - Choriocarcinoma (gestational or nongestational)
  - Germ cell tumors with trophoblast.

Sample: The fresh and first morning urine sample is preferred for testing. The urine should be clear and collected in a clean container (not to wash with soap). No preservatives should be added.

PREGNANCY TESTS

Pregnancy tests may be broadly classified as immunological or biological tests.

Immunological Tests for Pregnancy

There are different immunoassays for detecting HCG. Compared to biological methods, they are more rapid, simple, accurate, economical and easily standardized. They are broadly divided into:

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A. Immunoassays without radioisotopes
B. Immunoassays with radioisotopes

Immunoassays without Radioisotopes

Agglutination inhibition tests

Principle: It is based on latex particle agglutination inhibition. Latex particles coated with purified HCG serve as an antigen capable of reacting with an anti-HCG serum produced in rabbits (e.g. Gravindex test). It is a two stage procedure.

Procedure:

- One drop of patient's urine is mixed with one drop of a solution that contains HCG antibody (anti-HCG serum).
- Then one drop of another solution that contains latex particles coated with HCG is added.

Interpretation:

- **Positive test:** Test is considered as **positive** for HCG if there is **no agglutination** (homogeneous). If the patient is pregnant, HCG present in the urine will neutralize the antibodies in the antiserum, and added latex particles coated with HCG will not agglutinate.
- **Negative test:** Test is interpreted as **negative** if there **is agglutination**. If the patient is not pregnant (no HCG in the urine), the antiserum will not be neutralized and will agglutinate the latex particles.
  The quantitative estimation can be done by serial dilutions of the patient's urine (24-hour specimen).

Current pregnancy tests use immunoassays with monoclonal antibodies to measure human chorionic gonadotropin (HCG). Serum assays and sensitive urine assays can now detect pregnancy approximately 1 week after conception.

Direct agglutination test (HCG direct test)

Procedure: Latex particles coated with anti-HCG monoclonal antibodies are mixed with the urine sample to be tested.

Interpretation:

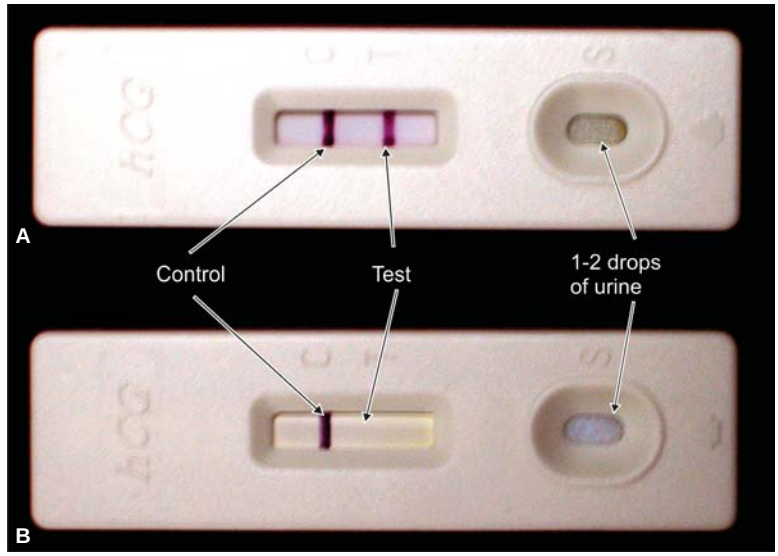
Positive test: Agglutination develops if the urine sample contains HCG and is considered as a positive pregnancy test.
Negative test: Absence of agglutination (urine without HCG) indicates that the test is negative for pregnancy.

Enzyme-linked immunosorbent assay (ELISA)

Principle: This test is based on binding of one monoclonal antibody with HCG and a second antibody that is linked with an enzyme to 'sandwich' the HCG. Presence of HCG is detected by a color change. This is more sensitive and specific. Various kits in card forms (card tests) are available (Figs 54.1A and B).

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Figs 54.1A and B: Card test showing: (A) both control and test band (pregnancy test positive): (B) only control band (pregnancy test negative)

Fluoroimmuno assay (FIA)

It is a highly precise sandwich assay. It uses a second antibody similar to ELISA, but is tagged with fluorescent dye. The fluorescence emitted is proportional to the amount of HCG. It is used not only to detect HCG, but also for follow up HCG concentrations.

Immunoassays with Radioisotopes

- **Radioimmunoassay (RIA):** This method employs radioactive isotopes (e.g. I^{125}) to label either the antigen or antibody. It is more sensitive and can detect very small quantity of β subunit of HCG (up to 0.002 IU/ml) in the serum.
- **Immuno-radiometric assay (IRMA):** This test uses sandwich principle to detect whole HCG molecule. IRMA uses I^{125} labeled HCG and is also very sensitive.

Biologic Pregnancy Tests

Biologic pregnancy tests employ animals. They are outdated and are of only historical interest.

Principle: Injection of HCG into certain animals produces characteristic anatomic changes either in ovary (production of corpora lutea, pronounced hyperemia) or secretion of sperm or ova in toads and frogs.

The biologic effect of the hormone and the sensitivity of different animal species vary greatly. In addition, amphibians may also show seasonal variations in sensitivity.

Various biological tests and their characteristics are presented in Table 54.1.

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Table 54.1: Different biological tests and their characteristics

Name of the test	*Animal used*	*Changes indicative of positive test*
Aschheim-Zondek (AZ) test	Immature, white female mice	Ovaries enlarged 2 to 3 times the normal size, presence of corpora lutea and/or grossly visible hemorrhagic follicles
Friedman test	Female rabbit	Hemorrhagic follicles and corpora lutea
Rat hyperemia test	Immature rats	At least one of the four ovaries is enlarged, red or covered with many red dots
Frog and toad tests	Male frogs or toads	Spermatozoa appear in the cloacal fluid after 1 to 4 hours

Causes of false negative:

- If the urine sample is allowed to stand for prolonged period at room temperature (8 hours or more), it may result in loss of HCG activity.
- Test done early in pregnancy may give false negative result, but it can be prevented by using fresh/first voided morning sample of urine.
- Ectopic pregnancy and threatened abortion are frequently associated with very low HCG levels.

SUMMARY

- Pregnancy test is employed for either qualitative or quantitative estimation of human chorionic gonadotropin hormone. Tests may be immunological or biological tests. There are several immunoassays which are rapid, simple, accurate and economical.

SELF-ASSESSMENT EXERCISES

I. Short Note

Pregnancy test

II. Multiple Choice Question

1. **Apart from pregnancy, pregnancy test is useful in**
   A. Endometrial tumors
   B. Gestational trophoblastic disease
   C. Surface epithelial tumors of ovary
   D. Sex cord stromal tumors of ovary

ANSWER

1. B

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CHAPTER 55 Sputum Examination

CHAPTER OUTLINE

- Introduction
- Examination of Sputum

INTRODUCTION

Sputum is a highly specialized watery, colorless and odorless product of the respiratory tract. Expectorated sputum is always abnormal and it consists of mucus and a variety of cellular and noncellular materials.

It is the most frequently received specimen from the respiratory tract. Both its collection and examination are advantageous as samples are easily obtained, cost effective and its cellular content is representative of the entire respiratory tract.

Sputum Collection

- The patient is instructed to cough up to get the sputum proper and the same is collected in a wide mouthed sterile, glass/plastic container with screw cap.
- In those patients who cannot produce sputum spontaneously by deep coughing, a specimen of sputum may be induced. This is done by inhalation of appropriate solvents which are aerosolized to stimulate sputum production.
- Early morning sputum sample is preferred for routine examination and 24 hours sample for the demonstration of tubercle bacilli by concentration method.

EXAMINATION OF SPUTUM

Sputum examination consists of (1) physical examination, (2) microscopic examination, and (3) culture study.

Physical Examination

- **Quantity**
  - In bronchiectasis, large amount of purulent sputum is coughed out.
  - Large amount of watery sputum with pink tinge suggests pulmonary edema.
- **Appearance/color:** Different colors of sputum and its causes are shown in Table 55.1.
- **Odor or smell:** Foul smelling sputum is observed in bronchiectasis and lung abscess and is due to anaerobic bacterial infections.

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Table 55.1: Different colors of sputum and its causes

Appearance/color	*Causes*
• White, viscid, mucoid	• Asthma
• Serous, clear, watery	• Pulmonary edema
• Frothy, pink, serous	• Bronchoalveolar carcinoma
• Clear or mucoid, gray, glassy, tenacious	• Chronic bronchitis
• Yellow due to pus/neutrophils	• Acute lower respiratory tract (pulmonary) infections
• Green (neutrophils which release green pigmented enzyme called verdoperoxidase)	• Long standing infection (bronchiectasis, lung abscess) e.g. pseudomonas
• Rusty due to lysis of red cells	• Pneumonia (e.g. pneumococcal) and pulmonary infarction
• Bright red due to fresh blood	• Pulmonary tuberculosis, lung tumors, pulmonary infarction
• Black due to coal dust	• Coal workers pneumoconiosis or in heavy smokers
• Anchovy sauce (chocolate brown)	• Rupture of amebic liver abscess into lung
• Blood tinged sputum	• Mitral stenosis, pulmonary tuberculosis, carcinoma lung, pulmonary infarction

Microscopic Examination

Staining of sputum: Two to three smears are made on a clean dry glass slides and are stained with:

- Leishman stain or Wright stain for differential count
- Other stains (depends on the clinical/pathological features)
- Gram's stain for microorganisms
- Ziehl-Neelsen stain for acid fast tubercle bacilli
- Special stains for fungi
- Papanicolaou stain for study of malignant cells (Chapter 56).

Cells

Normal sputum consists of a few neutrophils, few lymphocytes, carbon laden macrophages, occasional eosinophils and red cells. Various types of cells seen in sputum and their significance are shown in Table 55.2.

Table 55.2: Type of cells in sputum and their significance

Type of cell	*Significance*
Pus cells (neutrophils)	Numerous pus cells indicate pyogenic infection of the respiratory tract
Eosinophils	Increased eosinophils are seen in asthma and parasitic infections of the lungs
Lymphocytes	Increased lymphocytes are seen in early pulmonary tuberculosis
Red blood cells	If increased, indicate hemorrhage (bleeding) into the lungs or the bronchi
Heart failure cells (hemosiderin-laden macrophages)	Chronic venous congestion of the lungs, pulmonary infarction and pulmonary hemorrhage
Anthracotic pigment-laden cells (carbon-laden)	Increased in coal workers' pneumoconiosis and those who live in smoky polluted atmosphere

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Other Structures

- Curschmann spiral: They appear as spiral structures with a central thread found in the sputum of patients with bronchial asthma. Their exact nature is not known.
- Charcot-Leyden crystals: These appear as fine needle shaped or hexagonal colorless crystals, which are formed when the sputum is allowed to stand for some time. In bronchial asthma, they form due to the disintegration of eosinophils.

Parasites

- Larvae of *Strongyloides stercoralis* and roundworm: They may be found in sputum of patients with corresponding parasitic infestations.
- *Entamoeba histolytica*: Cysts or the trophozoites may be found when an amoebic liver abscess ruptures into the lungs.
- *Echinococcus granulosae*: Scolices and hooklets of the larval form may be seen with the rupture of the hydatid cyst of the lungs into the bronchus.

Culture Study

Sputum culture may demonstrate the causative infectious agent.

SUMMARY

- Sputum is a highly specialized product of the respiratory tract and expectorated sputum is always abnormal.
- Sputum examination consists of physical, microscopic examination and culture.
- Microscopically, sputum smears are stained by Leishman stain (cell type), Gram's stain (for microorganisms), Ziehl-Neelsen stain (for acid fast tubercle bacilli) and Papanicolaou stain (for study of malignant cells).

SELF-ASSESSMENT EXERCISES

I. Short Note

Sputum examination and its importance

II. Multiple Choice Question

1. **Rusty sputum is characteristically seen in**
   A. Bronchiectasis
   B. Trauma to the lung
   C. Pneumococcal pneumonia
   D. Carcinoma lung

ANSWER

1. C

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CHAPTER 56

Cytology

CHAPTER OUTLINE

- Introduction
- Cytology of Female Genital Tract
- Cytology of Other Systems
- Buccal Smear for Barr Body
- FNAC Appearance of some Common Lesions

INTRODUCTION

Definition: Cytology is the study of individual cells and/or tissue fragments.

Uses of cytology:

- **Diagnosis**
  - The diagnosis of benign, dysplastic and malignant lesions.
  - Intraoperative diagnosis
  - The diagnosis of inflammatory lesions and specific infections (e.g. tuberculosis)
- **Assessment of hormonal status** in women
- **Cytogenetics, DNA cytometry** and **DNA ploidy analysis**

Steps Involved in Processing of Cytological Smears

Three steps are involved namely: (1) specimen collection, (2) fixation and (3) staining of smears.

Specimen Collection

The cytological sampling techniques to obtain the cells for study may be divided into five main groups.

- **Exfoliative cytology:** It is the study of cells which shed off spontaneously/**exfoliated (shed)** from the lining of an organ into a body cavity. The principle of exfoliative cytology is that, the cells normally exfoliate from any surface lining and this exfoliation increases in pathological conditions.
  Source: The different sources of exfoliated cells are:
  - **Surface of mucosal or epithelial lining**
    - **Female genital tract**: Cervix, vagina
    - **Respiratory tract:** Sputum
    - **Urinary tract:** Urine

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- **Body fluids**
  - **Effusions:** Pleural, peritoneal and pericardial
  - **Other fluids:** Synovial fluid and CSF

Uses: Exfoliative cytology is useful for the diagnosis of various diseases, identification of dysplasia and malignancy. The malignant cells become less cohesive and are shed easily.

- **Abrasive cytology:** In abrasive cytology viable cells are artificially obtained by **abrasive techniques** from the surface of an organ. The instrument used to obtain the cells includes **scraper or spatula** (cervical, oral cavity-buccal smear for sex chromatin) and cytological brush (bronchial, gastrointestinal tract). The cells may also be obtained by **washing or lavage** (peritoneal, bronchoalveolar), where small amount of saline or similar solution is instilled into the target organ and aspirated. The cells obtained by abrasive technique do not show degenerative changes or necrosis, in contrast to that of exfoliative cytology.
- **Fine needle aspiration cytology:** By this technique, cells are obtained from various organs or masses by needle aspiration (**fine needle aspiration cytology-FNAC).** FNAC is the most widely and commonly used procedure for the assessment of localized and readily palpable lesions of different organs or sites. It is less invasive, rapid, extremely reliable and useful procedure which can be performed on an outpatient basis. In FNAC, small amount of tissue/cells are aspirated with a small-bore needle (22G/23G) using Franzen handle (Fig. 56.1). Aspirated material is spread on the slide and smears are stained with Pap/ Giemsa/H&E stain.



Fig. 56.1: Franzen handle with syringe and 22G needle

Common sites for FNAC

- Lymphnodes
- Breast
- Salivary glands
- Thyroid
- Soft tissue masses
- Intra abdominal masses under ultrasound/CT guidance (kidney, liver, etc).

Modern imaging techniques have extended its use to lesions in deep-seated structures like pelvic lymph nodes, pancreas and lungs.

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- **Intraoperative cytology:** Procedure by which the cells are obtained during operation, which includes:
  - **Touch (imprint) cytology:** Smears are prepared by forcefully pressing the clean glass slide to the cut surface of the organ.
  - **Scrape cytology:** Smears are prepared from material obtained by scraping the cut surface of the biopsy specimen with clean scalpel.
  - **Crush preparation (crush cytology):** Small fragments of fresh unfixed surgical tissue are crushed between two slides. This technique is mainly used in neuropathology.

  Uses: It can be used for obtaining cells from any organ or tissue. It supplements or replaces the frozen section.

  Advantage: Easier, cheaper and faster to prepare smears and useful for rapid diagnosis.

  Disadvantage: Interpretation of smears requires training and experience.
- **Liquid-based cytology (LBC):** It is a newer technology in which cells are immerzed in a conserving liquid before being fixed on the slide. This avoids desiccation and reduces the quantity of obscuring material. One of the methods is Thinprep Pap Test.

  Advantages:
  - Additional slides can be prepared from the liquid.
  - Molecular testing of infectious agents
  - DNA cytometry
  - DNA ploidy analysis

  Disadvantage: Higher cost.

Fixation of Cytological Smears

Different methods of fixation of cytological smears are:

- **Wet fixation:** Cytological smears are fixed immediately (when smears are wet) in any of the following fixatives in a coplin jar.
  - 95% alcohol
  - A mixture of equal parts of 95% alcohol and ether
- **Spray fixation:** In this method, the cytological smears are immediately fixed with a spray fixative. This type of fixative usually contains alcohol (e.g. hair spray).
- **Air dried smears:** Smears are air dried and fixed in 95% alcohol for 5 minutes (for May-Grunwald Giemsa stain).

Staining of Smears

Different stains that can be used for cytological specimen are: **Papanicolaou (Pap) stain**, **May-Grunwald Giemsa (MGG), hematoxylin and eosin (H&E)** and **Leishman stain**. Most commonly used stain is the Pap stain.

Papanicolaou (Pap) smear and Pap stain: Dr George N Papanicolaou (an American of Greek descent) is one of the pioneers who observed certain changes in cells of vaginal smears. He incidentally observed cancer cells in vaginal smears of women and the cytological technique was

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applied for the first time to discover the occult cancer of cervix. Papanicolaou's name was applied to cytologic procedure done for detection of the cervical cancer. Thus, the term **Pap** (cytologic) smear is usually applied to smears obtained from female genital tract. The Pap stain is also invented by Papanicolaou and bears his name.

Procedure for Pap Stain

- Take the smears which are fixed in any of the above mentioned fixatives and hydrate them by processing through descending grades of alcohol (80, 70 and 50%) to distilled water.
- Stain in Harris' hematoxylin for 4 minutes.
- Wash in running tap water 1-2 minutes.
- Dehydrate the smears up to 95% ethyl alcohol.
- Stain in OG-6 (Orange-G) for 1-2 minutes.
- Rinse the slide 10 times in 95% alcohol.
- Stain in EA-36 (Eosin A) for 2-3 minutes.
- Rinse the slide in 3 separate jars containing 95% alcohol.
- Dehydrate, clear and mount the slide.

Uses

- Staining of cervical smears (Pap smears) for diagnosis of carcinoma of the cervix (often at *in situ* stage).
- Smears from other organs with suspected malignancy (e.g. endometrial carcinoma, bronchogenic carcinoma, tumors of bladder and prostate and gastric carcinomas).
- For the identification of tumor cells in body fluids (abdominal, pleural, joint and cerebrospinal fluids).
- Assessment of hormonal status and inflammatory conditions.

CYTOLOGY OF FEMALE GENITAL TRACT

In the female genital tract, the cells can be obtained by different techniques depending on the purpose (e.g. hormonal assay). The different types of smears are:

- Lateral vaginal wall smear
- Cervical Smear
- Vaginal pool smear
- Endocervical and endometrial smears: These are not done routinely.

Lateral Vaginal Wall Smear

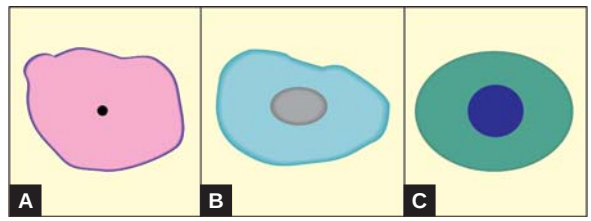
The cells are obtained by scraping the upper third of lateral vaginal wall and smears are prepared. It is suitable for cytohormonal assessment and is preferable to the posterior fornix pool smears. The cytological features and appearance of cells in normal vaginal smear are shown in Table 56.1 and Figs 56.2A to C, respectively.

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Table 56.1: Cells found in normal vaginal smears

Type of cell	*Morphological features*
Superficial cells	Mature, polygonal, eosinophilic cytoplasm with pyknotic nuclei
Intermediate cells	Polygonal, cyanophilic cytoplasm with vesicular nuclei
Parabasal cells	Small, oval, cyanophilic cytoplasm with large vesicular nuclei
Basal cells	Small, round to oval, deeply basophilic cytoplasm



Figs 56.2A to C: Normal superficial (A), intermediate (B) and parabasal (C) cells that are found in the vaginal smear

Uses of Vaginal Smear

- For detecting the following **inflammatory conditions** which may reveal either the causative agent or morphological features associated with them.
  - Trichomonas vaginalis
  - *Candida albicans*

  } These causative agents can be detected in the smears
  - Human papilloma virus (HPV)
  - Herpes simplex virus
- **Diagnosis of cervical intraepithelial neoplasms and malignancies**
- **For hormonal assessment**
  - **Maturation index (MI):** It represents a **differential count** of the different squamous cell populations. It is expressed as a percentage of parabasal, intermediate and superficial cells (e.g. 10/20/70).
  - **Karyopyknotic index (KI):** It is useful in assessing ovulatory/anovulatory cycles. It is the **percentage** of superficial squamous epithelial **cells having pyknotic nuclei** and expressed as percentage of total mature squamous cells.
  - **Cornification index: The percentage of acidophilic squamous cells** is termed as cornification index (CI) or eosinophilic index (EI).

Cervical Smear

The cells are obtained from cervical os by using an Ayre's spatula (wooden or plastic) which is rotated through 360°, so as to obtain the cell sample from both ectocervix and squamo-columnar junction. The scraped material on the Ayre's spatula (Fig. 56.3) is smeared on glass slides and smears are prepared. Smears are wet fixed, stained by Pap stain and are usually recommended for detection of dysplasia and carcinoma of cervix.

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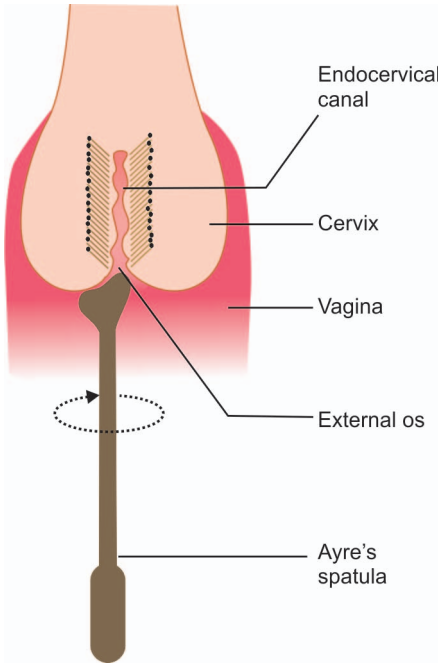


Fig. 56.3: Method of obtaining cervical smear by Ayre's spatula

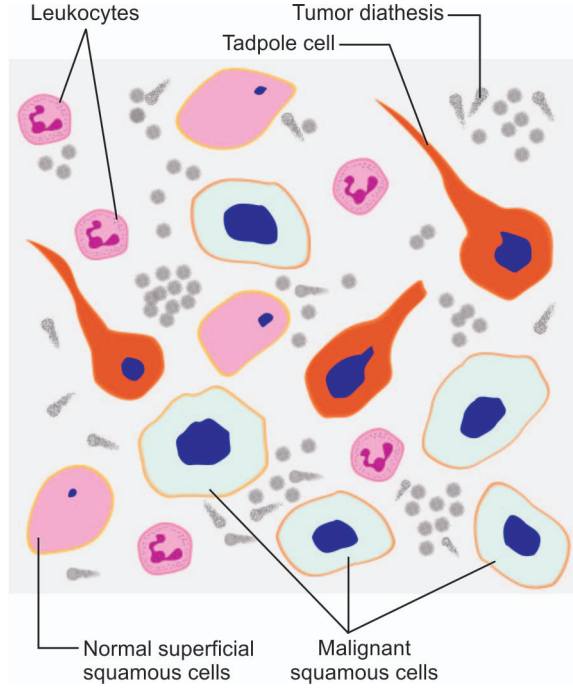


Fig. 56.4: Cytological appearance of squamous cell carcinoma of cervix

Bethesda (2001) system: Currently the cytological smears are reported according to the Bethesda (2001) system. According to this system, the cytological features of the precursor lesions of carcinoma cervix are divided into:

- LSIL (low grade squamous intraepithelial lesion)
- HSIL (high grade squamous intraepithelial lesion)

Squamous cell carcinoma cervix (Fig. 56.4): Characteristic cytological features of invasive squamous cell carcinoma are:

- Cells present singly, in sheets and syncytial aggregates.
- Marked variation in cell size and shape. Elongated and bizarre shaped malignant cells, tadpole/caudate cells and spindle squamous cells are often seen.
- Cytoplasm is eosinophilic in tadpole shaped cells.
- High nuclear cytoplasmic ratio.
- Nuclei have irregular membranes with coarse chromatin.
- Background shows tumor diathesis (necrosis and old degenerated blood).

Vaginal pool smear: Cells are obtained by aspirating material from the posterior fornix of vagina and smears are prepared on the glass slide.

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CYTOLOGY OF OTHER SYSTEMS

Cytology of Respiratory Tract

The source of cells from respiratory tract may be:

- Sputum
- Bronchosopy
  - Bronchial aspirates and washing
  - Bronchial brushing (BB)
  - Bronchoalveolar lavage (BAL)
- Fine needle aspiration including transbronchial fine-needle aspiration.
- Pleural fluid.

Sputum examination (Chapter 55)

- Cyotological examination of sputum is usually done for diagnosis of primary and metastatic malignancies of lung. At least three samples of sputum (preferably early morning samples) should be examined.
- The solid particles/blood specked areas from sputum are smeared over slides and stained with Papanicolaou stain.

Cytology of Gastrointestinal Tract

Cells from lesions of the oral cavity can be obtained by scraping the surface with a metal or wooden spatula. Cell samples from esophagus, stomach, small and large intestines can be obtained either by brushing or lavage during fibreoptic endoscopy.

Cytology of Urinary Tract

Cells from urinary tract lesions may be obtained from either urinary sediment prepared from voided urine/catheterized urine or bladder washings obtained at cystoscopy. The deposit is stained and examined. Specimen may also be obtained by aspirates and brushings.

Cytological examination of body fluids (Chapter 51) and CSF (Chapter 52) are discussed separately.

BUCCAL SMEAR FOR BARR BODY

Buccal smears for Barr body (Fig. 56.3) are prepared with a thin wooden spatula, by scraping the buccal mucosa. Smears are stained by Papanicolaou stain.

Barr body is seen as a solitary condensation on nuclear membrane in the intermediate squamous cells from buccal smears.

In normal females, Barr bodies are present in 4 to 20% of nuclei of squamous cells, where as in males, their count is less than 2% of nuclei.

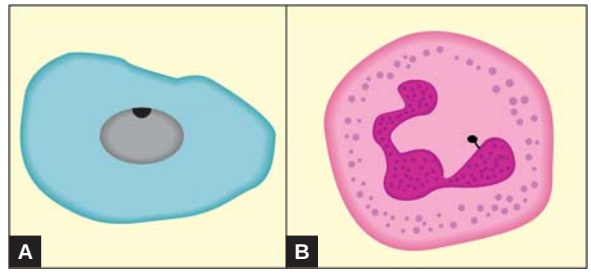
In XXX female, 2 Barr bodies are found while in **Turner syndrome (XO),** the **Barr body is absent.** In males with **Klinefelter syndrome (XXY)** the **Barr body is present**. Neutrophils in the peripheral smear may also be examined for nuclear sexing.

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Leukocytes: Nuclear Sexing (Figs 56.5A and B)

Abnormalities of sex chromosomes can be diagnosed by nuclear sexing. In a normal female (XX), the neutrophils in a peripheral smear show a drumstick which is counterpart of Barr body in buccal smear. Absence of drumstick is observed in *Turner syndrome* (XO), while one drumstick is found in males with *Klinefelter syndrome* (XXY).



Figs 56.5A and B: Nucleus of squamous cell with Barr body: (A) and neutrophil with drumstick (B)

FNAC APPEARANCE OF SOME COMMON LESIONS

Granulomatous (Tuberculous) Lymphadenitis (Fig. 56.6)

- Smears are cellular and composed of epithelioid cells in cohesive clusters forming granulomas.
- Epithelioid cells have elongated (oblong/slipper shaped) nuclei and abundant pale cytoplasm.
- Multinucleated Langhans type of giant cells may be seen.
- The background shows lymphocytes and eosinophilic granular caseous necrotic material.
- Ziehl-Neelsen stain for acid-fast bacilli may show positivity for the tubercle bacilli.

Pleomorphic Adenoma (Fig. 56.7)

- Epithelial cells in clusters, sheets and singly scattered.
- Regular ovoid nuclei with bland nuclear chromatin and well defined cytoplasm.
- Fibrillary chondromyxoid ground substance.
- Spindle shaped cells seen in stromal matrix.

Fibroadenoma Breast (Fig. 56.8)

- Moderately cellular smears.
- Large, branching monolayered sheets of uniform benign ductal epithelial cells having **antler horn** configuration.
- Numerous single bare bipolar nuclei of benign type.
- Fragments of loose fibromyxoid stroma.

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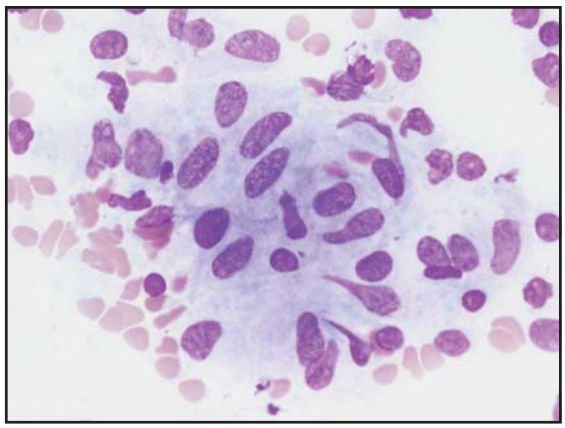


Fig. 56.6: Tuberculous lymphnode aspirate with granuloma composed of epithelioid cells showing slipper shaped nuclei and abundant pale cytoplasm

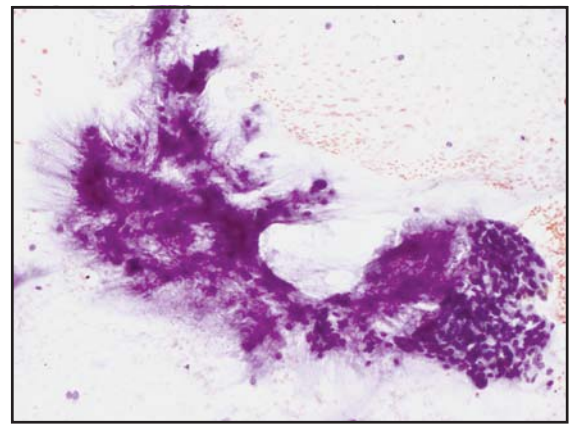


Fig. 56.7: FNAC of pleomorphic adenoma of salivary gland composed of sheets of epithelial cells in a fibrillary myxoid stroma

Infiltrating Duct Carcinoma Breast (Fig. 56.9)

- Highly cellular smear consisting of clusters of loosely cohesive malignant cells of variable size and shape.
- High nuclear cytoplasmic ratio; nuclei are enlarged, hyperchromatic with irregular nuclear membranes.
- Absence of single bare nuclei of benign type.
- The background may show necrosis.

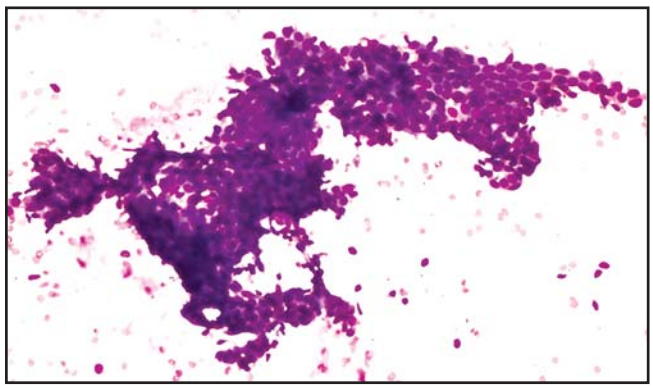


Fig. 56.8: FNAC breast of fibroadenoma consisting of monolayered sheets of benign ductal cells with antler horn configuration

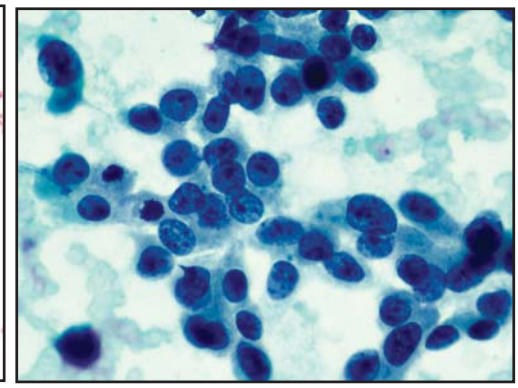


Fig. 56.9: FNAC breast showing malignant cells in infiltrating duct carcinoma

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SUMMARY

- Cytology is the study of individual cells or tissue fragments.
- Cytology is useful for diagnosis, assessment of hormonal status in women and cytogenetic analysis.
- Study of cells which shed off spontaneously is known as exfoliative cytology.
- FNAC is the most widely and commonly used procedure for the assessment of localized and readily palpable lesions of different organs or sites.
- Liquid-based cytology is a newer technology in which cells are immersed in a conserving liquid before being fixed on the slide and has certain advantages over conventional cytology.
- Papanicolaou's name was applied to cytologic procedure done for detection of cervical cancer and the term Pap smear is usually applied to smears obtained from female genital tract and the stain also bears his name.
- Cervical smears are obtained by using an Ayre's spatula and are reported by using Bethesda (2001) system.

SELF-ASSESSMENT EXERCISES

I. Short Notes

1. Fine needle aspiration cytology
2. Exfoliative cytology-indications and importance
3. Pap smear
4. Application of Papanicolaou (Pap) staining
5. Fixatives in cytology
6. Barr body

II. Multiple Choice Questions

1. **Common fixative used in cytology is**
   A. 50% alcohol
   B. 95% alcohol
   C. 10% formalin
   D. 40% formaldehyde
2. **For cytohormonal assessment the preferred site of obtaining the cells is**
   A. Anterior fornix
   B. Posterior fornix
   C. Upper third of lateral vaginal wall
   D. Lower third of lateral vaginal wall
3. **The epithelial cells with antler horn configuration are characteristically observed in FNAC of**
   A. Infiltrating duct carcinoma
   B. Pleomorphic adenoma
   C. Fibroadenoma
   D. Tuberculous lymphnode

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4. **Bethesda (2001) system is used for reporting**
   A. Buccal smears
   B. Crush smears
   C. Cervical smears
   D. Ultrasound guided FNAC smears
5. **Tadpole cells are seen in**
   A. Carcinoma *in situ*
   B. Infection by candida
   C. Trichomonas vaginalis infection
   D. Squamous cell carcinoma of cervix
6. **Barr body count is less than 2% of nuclei of buccal mucosal squamous cells in**
   A. Male
   B. Postmenopausal female
   C. Klinefelter syndrome
   D. Girl younger than 10 years

ANSWERS

1. B **2.** C **3.** C **4.** C **5.** D **6.** A

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CHAPTER 57 Glucose Tolerance Test

CHAPTER OUTLINE

- Oral Glucose Tolerance Test
- Glycosuria

ORAL GLUCOSE TOLERANCE TEST

The glucose tolerance test (GTT) is also known as the oral glucose tolerance test which measures the body's ability to metabolize glucose.

Indication: It is performed for the **diagnosis of diabetes** in patients with borderline fasting blood glucose level (i.e. between 100 to 140 mg/dL).

Preparation of Individual

Patient is advised to take carbohydrate-rich diet for at least 3 days prior to the GTT. The patient should be in overnight fasting of at least 10 hours. Drinking water is allowed during the fasting period.

Procedure

- A fasting blood sample and urine sample is collected for estimation of the fasting blood glucose value. It provides a baseline for comparing other glucose values.
- Patient is given 75 g glucose (amount varies with weight/age) orally, dissolved in about 300 mL of water.
- Blood and urine samples are collected at timed intervals of 30 minutes for at least 2 hours after drinking glucose.
- Blood samples are analyzed for glucose level and urine samples are qualitatively tested for glucose.

Amount of Glucose to be Given in OGTT

- Standard OGTT uses 75 gm of glucose.
- In children glucose should be given based on the weight (1.5 to 1.75 gm/kg).
- In pregnant women, 100 gm oral glucose is recommended.

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Interpretation (Fig. 57.1)

- ***Normal/euglycemic:*** On taking oral glucose load, the concentration of blood glucose increases and peak value (140 mg/dL) is reached in less than an hour which returns to normal by 2 hours. None of the urine sample shows glucose.
- ***Prediabetics/impaired glucose tolerance:*** In patients with impaired glucose tolerance, both the fasting (110 to 126 mg/dL) and 2 hour (140 to 200 mg/dL) blood glucose levels are increased. These patients develop frank diabetes later.

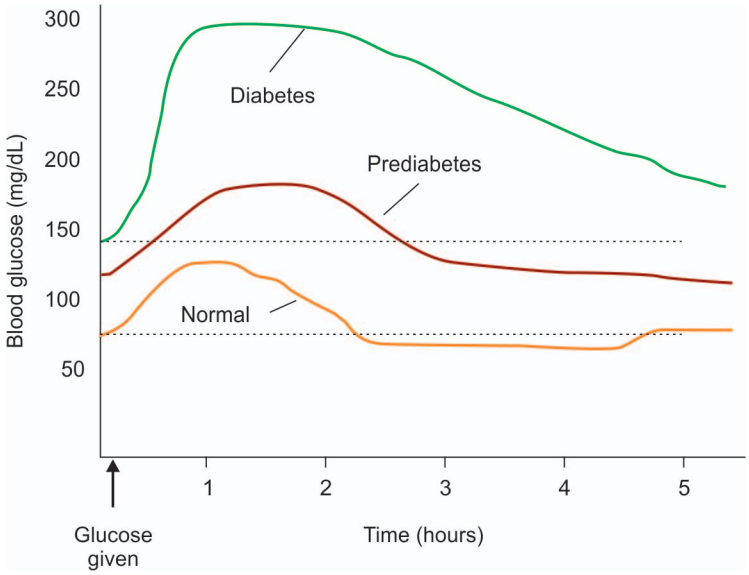


Fig. 57.1: Oral glucose tolerance test showing variation of blood glucose level in normal, prediabetes and diabetes mellitus

- ***Diabetes mellitus:*** The individual is diagnosed as suffering from diabetes mellitus if the fasting blood glucose levels are more than 126 mg/dL and at 2 hours more than 200 mg/dL. Diagnostic criteria for oral glucose tolerance test are shown in Table 57.1.
- ***Gestational diabetes:*** It is defined as any degree of glucose intolerance developing during pregnancy. About 2 to 5% of females with gestational diabetes mellitus carry mutation in the glucokinase gene.

Table 57.1: Levels of blood glucose in normal, prediabetes and diabetes in oral glucose tolerance test

Time	*Blood glucose concentration*		
	Normal/euglycemic	***Prediabetes/****Impaired glucose tolerance*	***Diabetes***
Fasting glucose level	Less than **100** mg/dL	Greater than **100** mg/dL but less than **126** mg/dL	Greater than **126** mg/dL
2 hours after taking glucose—OGTT	Less than **140** mg/dL	Greater than **140** mg/dL but less than **200** mg/dL	Greater than **200** mg/dL

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Other Types of GTT

- In the **mini GTT,** fasting and 2 hours samples (instead of ½ hr interval) of blood and urine are collected.
- In individuals with malabsorption, **intravenous GTT** is carried out.
- **Glucosteroid stressed GTT** is employed to detect latent diabetes.

The GTT is unphysiological way of evaluating the glucose handling by the body. Physiological way is by collecting the **fasting blood** sample and the individual is allowed to take heavy breakfast. The blood and urine samples are collected at 1 hour and 2 hours (**postprandial**—meaning after meal). This is commonly employed in established diabetic patients for monitoring the control.

GLYCOSURIA

- Normally glucose does not appear in urine until the plasma glucose concentration exceeds renal threshold (180 mg/dL). The presence of glucose in urine is known as glycosuria. The commonest cause of glycosuria is diabetes mellitus and therefore, urine examination for glucose is the first line screening test for diabetes.
- ***Renal glycosuria:*** Renal glycosuria is a benign condition due to a reduced renal threshold for glucose. It is unrelated to diabetes and not accompanied by the classical symptoms of diabetes. Therefore, it should not be mistaken as diabetes mellitus.
- ***Alimentary glycosuria:*** In certain individuals, blood glucose level may rapidly increase after meals resulting in its spill over into urine. This condition is known as alimentary (lag storage) glycosuria. It may be seen in some normal individuals and some patients with hepatic diseases, hyperthyroidism and peptic ulcer.

SUMMARY

- The glucose tolerance test (GTT) measures the body's ability to metabolize glucose and helps in the diagnosis of diabetes.
- The fasting blood sugar and urine are examined before giving the oral glucose, the dose of which depends on the weight of the individual and is usually about 75 gm in an adult.
- The blood and urine samples are collected at every half an hour at least for 2 hours.
- In diabetes mellitus, the fasting blood glucose levels are more than 126 mg/dL and at 2 hours more than 200 mg/dL.

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SELF-ASSESSMENT EXERCISES

I. Short Note

Glucose tolerance test

II. Multiple Choice Question

1. **The following is NOT true with regard to renal glycosuria**
   A. It is a benign condition
   B. These patients develop frank diabetes later
   C. It is due to a reduced renal threshold for glucose
   D. It is not accompanied by the classical symptoms of diabetes

ANSWER

1. B

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CHAPTER 58 Blood Group System

CHAPTER OUTLINE

- Introduction
- ABO Blood Group System
- Rh Blood Group System
- Other Blood Group Systems

INTRODUCTION

More than 400 red blood cell antigens have been identified, most of which are inherited in a Mendelian dominant fashion and only few are clinically important. These antigens form more than 20 genetically determined blood group systems.

The two most important blood group systems are ABO and Rh systems.

ABO BLOOD GROUP SYSTEM

ABO Antigens

According to the ABO blood group system, there are four different kinds of blood groups: A, B, AB or O. The antigen on red cells may be A/B/AB or no antigen at all. There are two subgroups in group A, namely A_1 and A_2. A and B antigens are inherited as per the Mendelian laws. Each individual inherits two ABO genes, one from each parent and these genes determine the ABO antigens on their red blood cells. Absence of both antigens, A and B on the red cells is seen in blood group O.

ABO Antibodies

Natural ABO Antibodies

The serum contains naturally occurring antibodies against red cell A/B/AB antigens (Table 58.1). These are of IgM type and do not cross the placental barrier.

Table 58.1: ABO phenotypes ("blood groups") and genotype

Phenotype (blood group)	*Antigens on RBCs*	*Antibodies in serum*	*Genotypes*
A	**A**	Anti-B	*AA* or *AO*
B	**B**	Anti-A	*BB* or *BO*
AB	**A** and **B**	Neither	*AB*
O	Neither	Anti-A and Anti-B	*OO*

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The serological typing indicates the phenotype and family studies indicate the genotype. Genotype refers to the genes present and the phenotype is the red cell antigen on the cell surface.

Acquired ABO Antibodies

- An individual lacking a particular antigen may develop an antibody after (acquired) exposure to red blood cells carrying the corresponding antigen.
- Exposure occurs due to parenteral introduction of red cell antigens. This may develop during:
  - Transfusion of red cells: These acquired antibodies cause hemolytic transfusion reaction.
  - Pregnancy: Passage of fetal red cells (having paternal antigens foreign to the mother) into the maternal circulation during pregnancy may result in mainly IgG type of antibodies. They can cross the placental barrier resulting in hemolytic disease of the newborn. The most important acquired *antibody* is anti-D, which is a major cause of hemolytic disease of the newborn.

H Genes and H Antigens

The expression of A and B antigens are dependent on the presence of H gene. Most of the individuals are homozygous for the H gene. The sequence of events in the formation of A and B red cell antigens is:

- A and B red cell antigens are glycoproteins and their formation starts with basic precursor substance (Fig. 58.1).

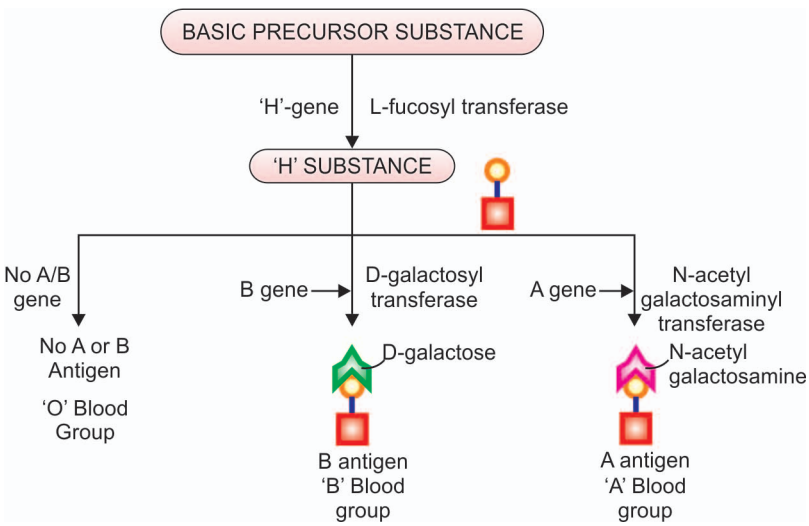


Fig. 58.1: Role of H gene in the formation of A and B antigens

- Basic precursor substance is first converted into H substance (by transferase) under the influence of H gene.
- H substance is partially converted under the influence of A and B genes (and specific transferase) into A and B antigens. Some of the H substance remains unconverted.

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- Since O group individuals do not have A and B genes, neither A nor B antigen is formed and these have only H substance (H substance remains unchanged).

Note: A, B and H antigens may be detected in the saliva and other body fluids also. Such individuals are called **secretors,** while the remaining without it are **nonsecretors.**

Bombay Blood Group

In 1952, Bhende, Bhatia and Deshpande discovered a new blood group known as Bombay blood group (phenotype Oh). These individuals lack the H gene and therefore the basic precursor substance cannot be converted into H substance. This in turn results in failure to form A or B antigen. When their blood sample is tested for routine ABO grouping, they will be labeled as blood group O. However, their serum contains anti-A, anti-B and anti-H antibodies. These individuals therefore, should be transfused with only Bombay blood group.

ABO System and Disease

- Group O individuals have increased risk of developing peptic ulcer.
- Group A individuals have increased risk of developing gastric carcinoma.

ABO Grouping Technique

ABO grouping is carried out by making a 2% saline suspension of red cells and adding anti-A, anti-B and anti-AB sera.

The different methods available are:

- Slide or tile technique
- Tube technique
- Microplate method
- Microtyping system
- Automated or semi-automated method.

The slide technique is easier to do and is described below.

Slide or Tile Technique

- Take a slide/white tile and mark it anti-A, anti-B and anti-AB.
- Put one drop each of anti- A, anti-B and anti-AB sera on the marked slide.
- Add one drop of washed 2% red cell suspension to each anti-sera.
- Mix each one separately with clean applicator stick and spread the mixture over an area of 2 cm.
- Rock the slide gently and look for "agglutination" within 5 minutes.

Interpretation: Blood group A, B, AB or O is interpreted depending on the agglutination in the anti-sera (Table 58.2 and Fig. 58.2).

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Table 58.2: Interpretation of ABO Grouping

Antisera added			*Interpretation*
Anti-A	*Anti-B*	*Anti-AB*	*Blood group*
–	–	–	O
+	–	+	A
–	+	+	B
+	+	+	AB

+ = Agglutination; – = No agglutination

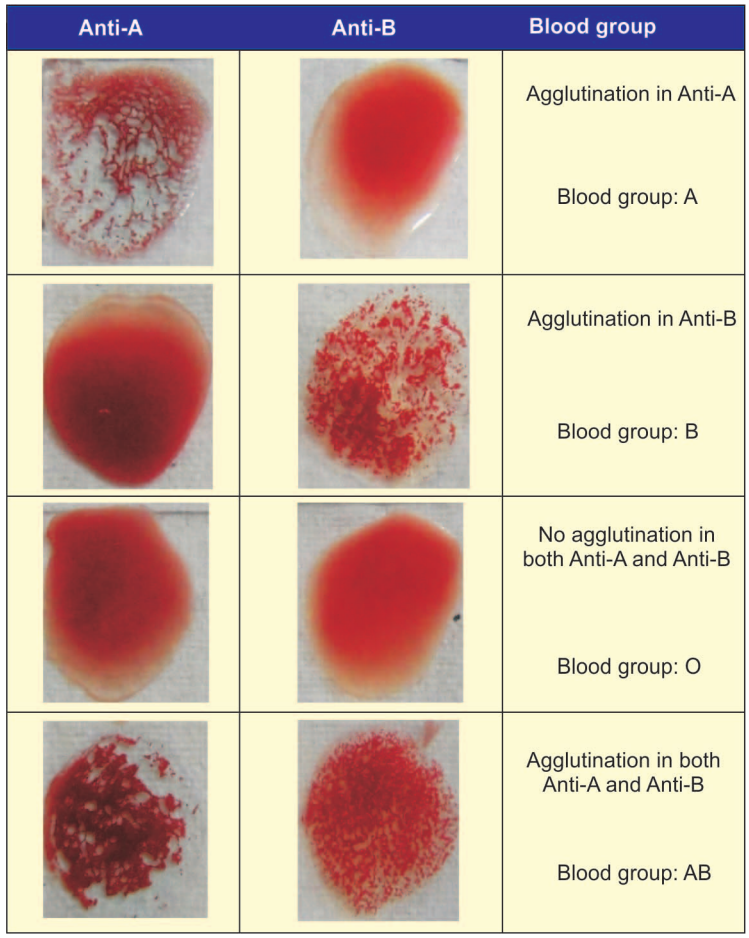


Fig. 58.2: Interpretation of blood group by slide method

Tube Technique

- Take 3 test tubes (75 × 10 mm size) and label them as anti-A, anti-B and anti-AB respectively.
- Add one drop each of the corresponding serum to each tube (i.e. anti-A, anti-B and anti-AB).
- Add 1 drop of 2% red cells suspension to each one of the three test tubes and mix them gently.
- Leave the tubes at room temperature for 90 minutes (or centrifuge for 15-20 minutes).

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- Look for agglutination.
- Transfer one drop of mixture from each tube onto a glass slide, using a thin bore Pasteur pipette. Spread the cells over an area of 2 × 1 cm, using the pipette.
- Examine the slide under microscope for "agglutination".

Interpretation: Blood group is interpreted depending upon the agglutination in the anti-sera (Table 58.2).

Controls

Each blood group test should preferably have controls, both positive and negative.

Positive control: This is run by performing the above techniques by using cells of known groups A, B, AB and O. This is especially useful for testing the potency of anti-sera.

Negative control: Run the above tests by adding saline instead of anti-sera. If agglutination develops it indicates auto agglutination or pseudo agglutination.

Microplate Method

Microplate method is ideal for testing large number of blood samples and has replaced test tube method in many laboratories. Microplates are polystyrene plates having 96 small well. It saves time and cost.

Microtyping System (Gel Card)

Microtyping system (MTS) is a gel based technology in which plastic cards contain microtubes filled with buffered dextran gel. The gel contains sodium azide (preservative) and group specific antisera (anti-A, anti-B, anti-AB, anti-D) or antiglobulin (for Coombs test). These constituents are incorporated into gel at the time of manufacture.

Technique

- Red cells are poured over the microtube.
- Incubate at 37°C and centrifuge.
- The cells settle down and react with the specific antisera (antibody) incorporated into the gel resulting in hemagglutination.

Interpretation

- **Positive test:** The gel matrix acts as a sieve and agglutinated red cells are too large to pass through the gel. Red cells are trapped at various places within the gel and form a line in the microtube indicating positivity of the test.
- **Negative test**: If the specific antigen is not present on the red cells, hemagglutination does not occur and the red cells pass through the gel forming a pellet at the bottom of the microtube.

Advantages

- Simple, safe, easy and cost-effective technique.
- Greater sensitivity when compared to conventional techniques.

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- Technical errors are eliminated.
- For Coombs test, red cells do not need saline washing.

Uses of microtyping system

- Blood grouping and pretransfusion cross matching.
- Coombs test
- For diagnosis of:
  - Syphilis, measles, diphtheria, parvovirus infection
  - PNH, sickle cell disease.

Note:

- A group has two subgroups namely A_1 and A_2, which can be differentiated by using anti-A_1 lectin.
- Presence of H substance can be detected by anti-H.

Rh BLOOD GROUP SYSTEM

Rh blood group system is the second system of clinical significance in transfusion medicine. This is a complete system of antigens which are labeled as D, d, C, c, E and e. The D antigen is highly immunogenic. Cc *and Ee antigens* are weak antigens and therefore risk of sensitization to these antigens is less than the risk of sensitization to D.

Rh (D) System

Rh Antigen

Rh positive (–ve)

Individuals who have D antigen on the red cell surface are Rh positive (+). This may be present as homozygous (DD) or heterozygous (Dd) state.

Rh negative (-ve)

Individuals who lack the D antigens are called as Rh-negative.

Rh Antibodies

- In contrast to ABO system, there are no naturally occurring antibodies against Rh antigens in Rh negative individuals.
- Acquired (immune) antibodies: Rh antibodies develop after exposure to Rh antigens either following transfusion (Rh +ve blood given to Rh –ve patients) or pregnancy (Rh +ve fetus in an Rh –ve mother). These antibodies are of IgG type and do not bind complement. They can be detected by antiglobulin test (Coombs test).

Clinical Significance

- Rh incompatibility between donor and recipient results in hemolytic transfusion reactions.
- Rh incompatibility between mother and fetus results in hemolytic disease of the newborn (HDN)

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Rh (D) Typing Techniques

The different methods available are:
- Slide or tile technique
- Tube technique
- Microplate method
- Microtyping System
- Automated or semi-automated method.

Slide or Tile Techniques

- Place one drop of anti-Rh (D) reagent (should be monoclonal IgM type) on a slide/white tile.
- Add 1 drop of 2% red cell suspension.
- Mix them using a clean applicator stick.
- Observe for agglutination after 2 minutes.

Interpretation: Presence of agglutination indicates that the blood sample is Rh +ve.

Tube Techniques

- Add one drop of anti-D to a small test tube (75 × 10 mm size).
- Add 1 drop of 2% saline red cell suspension.
- Mix them and incubate at 37°C for 30 minutes or centrifuge for 15 to 20 seconds.
- Look for agglutination.
- Transfer one drop of the mixture onto a glass slide, using a thin bore Pasteur pipette. Spread the cells over an area of 2 × 1 cm using the pipette.
- Examine the slide under microscope for "agglutination".

Interpretation: Presence of agglutination indicates Rh +ve blood group.

Controls

Known Rh +ve and Rh –ve samples should be run as positive and negative controls.

OTHER BLOOD GROUP SYSTEMS

These are weak antigens and have no clinical significance.
- Lewis system
- P system
- KELL system
- MNS system
- Duffy system
- Kidd system
- Lutheran system
- Yt system
- Dombrok system
- Colton system
- Diego system

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SUMMARY

- There are several blood group systems, important being ABO and Rh systems.
- Individuals with Bombay blood group lack the H gene and there is failure to form A or B antigen.
- Blood grouping can be performed by slide/tube/microplate/automated/semi-automated method or by microtyping system.
- Blood grouping is important before blood transfusion and is also of medicolegal value.

SELF-ASSESSMENT EXERCISES

I. Short Notes

1. ABO blood grouping system
2. Importance of blood grouping
3. Rh blood grouping

II. Multiple Choice Questions

1. The blood group in ABO system, that lacks antigen on the RBC membrane is
A. A
B. AB
C. O
D. B

2. The serum of an individual with B blood group contains
A. Anti-A
B. Anti-B
C. Anti-A and Anti-B
D. Anti-D

3. Bombay blood group is characterized by absence of
A. A gene
B. B gene
C. Both A and B gene
D. H gene

4. The most sensitive method for blood grouping is
A. Slide method
B. Tube method
C. Gel Card method
D. Microplate method

ANSWERS

1. C **2.** A **3.** D **4.** C

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CHAPTER 59

Antiglobulin Test

CHAPTER OUTLINE

- ❖ Direct Antiglobulin Test
- ❖ Indirect Antiglobulin Test

Antiglobulin (Coombs) test is useful to detect the presence of incomplete antibody (IgG) and/ or complement on the RBC membrane.

Principle: RBCs coated with incomplete antibody (IgG) or C3 complement does not cause agglutination of RBCs. Coombs reagent contains antibodies (antiglobulins) against human IgG/ IgM/complement. If the RBCs coated by incomplete antibody or complement, are treated with Coombs reagent, the antiglobulins in the reagent will induce agglutination of such RBCs. The standard Coombs reagent will not detect IgA or IgE antibodies.

Types of antiglobulin test: There are two types, namely direct and indirect antiglobulin tests.

1. **Direct** (Coombs) **antiglobulin test (DAT)**
2. **Indirect** (Coombs) **antiglobulin test (IAT)**

DIRECT ANTIGLOBULIN TEST

Direct antiglobulin test (DAT) (direct Coombs test) detects antibodies (IgG) and/or complement coated on the surface of patient's RBC membrane. **Patient's red cells are used in this test**.

Materials Required

- Patient's RBCs
- Coombs reagent
- Normal saline.

Technique (Fig. 59.1)

- Patient's RBCs are obtained from either the clotted fresh blood or blood sample in EDTA/ citrate.
- Take RBCs in a test tube and wash three times in normal saline.

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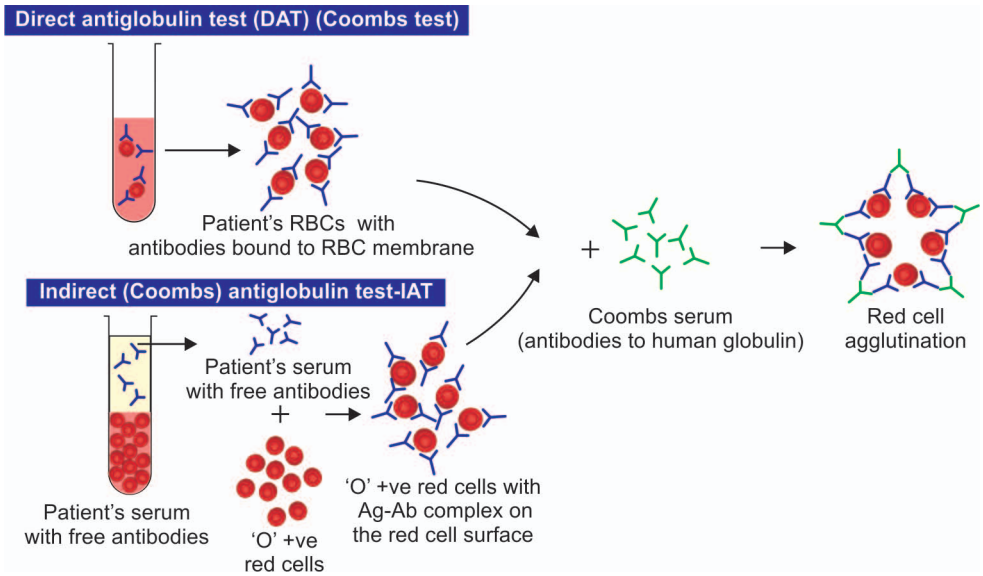


Fig. 59.1: Method of direct and indirect methods of antiglobulin test (Coombs test)

- Centrifuge (2500 rpm for 5 minutes) and decant the saline. Prepare a 5% saline suspension of RBCs.
- Take 2 drops of 5% saline red cell suspension in a test tube and add 2 drops of Coombs (antiglobulin) reagent.
- Mix well and incubate the test tube at 37°C for 5 minutes.
- Centrifuge at 1000 rpm for 1 minute.
- Gently shake the tube and observe for agglutination.

With each test, run **one positive control** and **one negative control.**

Interpretation

Agglutination indicates the presence of antibody on the RBC membrane and interpreted as positive DAT.

Uses of Direct Antiglobulin Test

- **Hemolytic disease of newborn** (HDN) in which direct Coombs test is performed on the newborn baby's red cells from the cord blood.
- **Autoimmune hemolytic anemia:** To demonstrate *in vivo* attachment of antibodies to red cells.
- Drug induced red cell sensitization
- Investigation of hemolytic transfusion reaction

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INDIRECT ANTIGLOBULIN TEST

Indirect antiglobulin test (IAT) (indirect Coombs test) detects the presence of incomplete (IgG) antibodies and/or complement in the patient's serum. **Patient's serum is used for this test.**

Materials Required

- Patient's serum
- O Rh positive RBCs
- Coombs reagent
- Normal saline.

Technique (Fig. 59.1)

- Take 2 drops of patient's serum in a test tube.
- Add 3 drops of 5% "O" Rh +ve cell suspension of any normal individual.
- Incubate the above mixture at 37°C for 10 minutes. During this time the "O" Rh positive RBCs are coated with (lgG) anti-Rh antibodies (if present) in the patient's serum.
- Wash with normal saline three times to remove the excess of antibodies. Remove the supernatant.
- Add 2 drops of Coombs (antiglobulin) reagent to the test tube and incubate at 37°C for 10 minutes.
- Centrifuge at 2000 rpm for 1 minute and examine for agglutination.

With each test, run **one positive control** and **one negative control.**

Interpretation

Agglutination of RBCs indicates the presence of antibodies in the patient's serum and test is reported as positive for indirect antiglobulin test.

Uses of Indirect Antiglobulin Test

- **Hemolytic disease of newborn:** Mother's serum is tested to detect anti-Rh antibody.
- **Cross-matching for blood transfusion:** To detect incompatibility of recipient's serum with donor's cells.

Controls for Antiglobulin Test

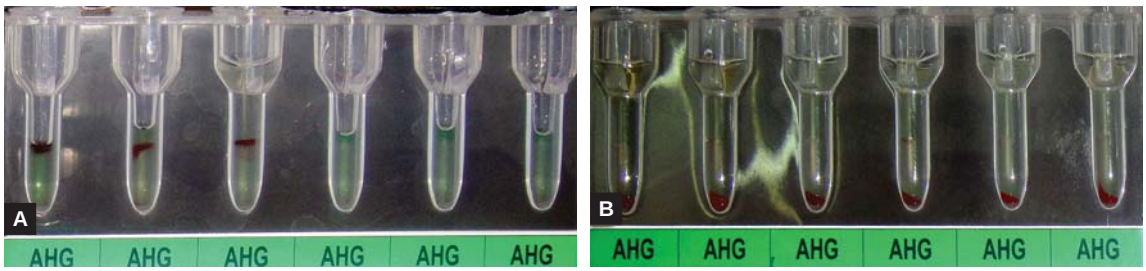
- ***Positive control:*** Sensitized (coated by anti-D) O Rh (D) positive cells.
- ***Negative control:*** Normal individual's RBCs which are nonsensitized.

Gel Card Method (Figs 59.2A and B)

This is **more sensitive** for detecting antibodies than conventional Coombs test.

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Figs 59.2A and B: Gel Card method of Coombs test. Positive: (A) Test shows agglutination and negative: (B) Test without any agglutination

SUMMARY

- Coombs test detects incomplete antibodies (IgG) and/or complements against the RBC membrane.
- There are two types of Coombs test, namely direct and indirect.
- In direct Coombs test (DAT), patient's red cells are used and it is positive in HDN (fetal red cells) and autoimmune hemolytic anemias.
- In indirect Coombs test (IAT), patient's serum is tested. It is useful in HDN where the mother's serum is tested for the presence of antibodies. It is also performed while cross-matching for blood transfusion, to detect incompatibility of recipient's serum with donor's cells.

SELF-ASSESSMENT EXERCISES

I. Short Notes

1. Antiglobulin (Coombs) test
2. Direct Coomb test procedure and conditions in which it is positive

II. Multiple Choice Question

1. In hemolytic disease of the newborn, the serum of mother is tested for Rh antibodies by
   A. Direct antiglobulin test
   B. Indirect antiglobulin test
   C. Cross matching with newborn blood
   D. AB, Rh positive red cells

ANSWER

1. B

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CHAPTER 60 Transfusion Medicine

CHAPTER OUTLINE

- Blood Transfusion
- Blood Components
- Transfusion Reactions

Transfusion medicine comprises of blood and blood component transfusion. Blood cannot be synthesized artificially. So, the source of blood is from a healthy human donor.

BLOOD TRANSFUSION

Blood transfusion is the process of transferring blood/blood products from donor into the circulating system of recipient. It is important to properly collect the blood from donor, prepare its components (if required) and store blood/components in a proper way and transfuse in such a way to avoid any risks or hazards.

Donor Selection

Donor selection is based on medical history and few routine physical examinations (weight, blood pressure, temperature, hemoglobin) are done to know whether donor is suitable for donating blood.

- Donor should be healthy. There are three types of donors namely voluntary (should be encouraged), replacement and professional.
- It is important to know whether the patient has history of diseases like hepatitis, AIDS, syphilis and if so, blood should not be obtained from them. In the blood bank, blood is routinely screen for few common infections.

Collection of Blood

- Blood is collected under aseptic conditions using sterile, plastic bag with anticoagulant (Fig. 60.1). Now CPD-A is used as an anticoagulant.
- Mix the blood and anticoagulant gently and periodically during its collection.

Anticoagulants used: The different anticoagulants—preservative solutions available are (Table 60.1)

- Citrate phosphate dextrose (CPD)
- Citrate phosphate dextrose adenine (CPDA-1)
- Acid citrate dextrose (ACD) is not used nowadays.

Storage of blood: Blood is stored in a refrigerator at 2° to 6°C.

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Fig.60.1: Triple plastic bag for blood collection

Table 60.1: Functions of various chemicals used in anticoagulant—preservative solution

Chemical used	*Function*
Dextrose	Needed for ATP generation for viability of red cells
Adenine	Substrate for red cell synthesis of ATP
Citrate	Prevents clotting by chelating calcium
Sodium biphosphate	Buffers the end product of glycolysis (lactic acid)

Predonation check-up

Donor Blood: The following tests are routinely carried out on donor's blood.

- ABO and Rh grouping
- Tests for:
  - HBsAg, anti-HCV, anti HIV-1 and HIV-2 and serum alanine aminotransferase (ALT)
  - Malaria and syphilis.

Recipient Blood: The recipient's ABO and Rh grouping is also carried out.

Compatibility Testing (Pretransfusion Testing)

Before transfusion of any blood or its components, it is essential to know whether they are compatible with the recipient's blood**.** This is achieved by performing a set of procedures known as compatibility testing. Sometimes the term compatibility test and cross matching are used interchangeably, but cross-match is only a part of compatibility test.

Compatibility tests include:

- Review of patient's past blood bank history and records (if done earlier).
- ABO and Rh typing of the recipient and donor.

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- Antibody screening test of recipient's and donor's serum.
- Cross-matching.

Cross-matching (Tables 60.2 and 60.3)

Cross-matching is very important before any blood transfusion. Cross-matching should be carried out to ensure that there are no antibodies in patient's serum that will react with the donor's cells when transfused.

Table 60.2: Types of cross-match

Type of cross-match	*Donor's*	*Recipient's (patient's)*
Major cross-match	Red cells	Serum
Minor cross-match	Serum	Red cells

Table 60.3: Major cross-match techniques

Type of cross-match	*Detects antibodies of type*
Saline cross-match	IgM
Albumin technique	IgG
Enzyme technique	IgG and some IgM
Antiglobulin test or Coombs test	IgG

Importance of cross-matching

- It is the final check of ABO compatibility between donor and recipient.
- Detects the presence of any clinically significant, unexpected antibodies in the recipient's serum that may react with donor's cells, thereby preventing any transfusion reaction.

A thorough cross-matching is performed with recipient's serum and donor's red cells.

Types

Cross-matching procedure may be divided into two major parts, namely **major and minor** cross-matching. Major cross-matching consists of mixing donor's red cells with recipient's (patient's) serum; whereas minor cross-matching consists of mixing patient's red cells with donor's serum (Table 60.2).

Major cross-matching: The simplest method is:

- Place one drop of patient's serum in a prelabeled glass test tube.
- Add one drop of 5% saline suspension of donor's red cells to the above test tube.
- Mix them and incubate at 37°C for 30 minutes. Centrifuge at 1000 rpm for 1 minute.
- Examine the tube for agglutination or hemolysis.

Interpretation

- If there is **no agglutination or hemolysis** it indicates compatibility.
- **Agglutination or hemolysis** indicates ***mismatching*** and recipient should not be transfused with the tested donor blood/component.

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Minor cross-matching: The above procedure may also be carried out by taking one drop of donor's serum and one drop of 5% saline suspension of recipient's red cells. Incubate at 37°C for 30 minutes. Centrifuge at 3000 rpm for 1 minute. Observe for agglutination or hemolysis.

It is always necessary to monitor the recipient during and after transfusion so that any complications can be dealt accordingly.

BLOOD COMPONENTS (FIG. 60.2)

It is possible to separate different components of blood from a single unit of whole blood. These components can be used individually to help more than one patient with many purposes. Thus, red cells can be transfused to an anemic patient and plasma for a burns patient. This also ensures that only the required components are transfused. The various blood components are:

- Red cells concentrate or packed red blood cells (PRBCs)
- Platelet concentrates
- Plasma
- Fresh frozen plasma (FFP)
- Cryoprecipitate

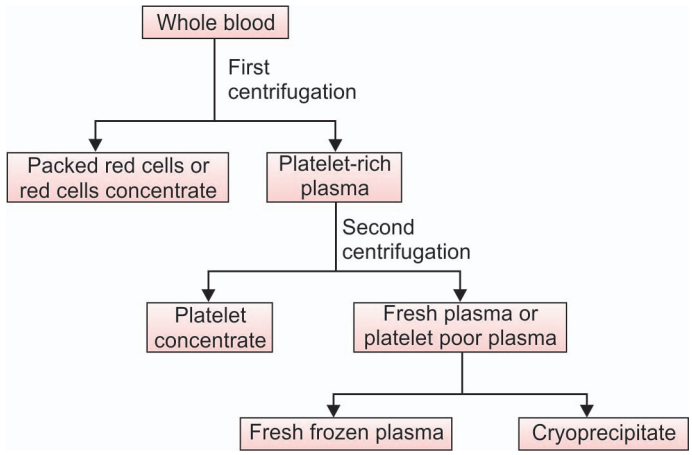


Fig. 60.2: Blood components

Red Cells Concentrate or Packed Red Blood Cells (PRBCs)

Packed red cells are obtained by centrifugation/sedimentation of the whole blood and the plasma is transferred to another bag. In the packed red cell unit, the hematocrit is between 55 to 65%. Since, the volume is only 200 ml; there is less risk of volume overload. One unit of packed red blood cells raises hemoglobin concentration by 1 gm/dL. These are stored in a refrigerator/cold room at 2° to 4°C and its shelf-life is one month.

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Indications

- Anemia
  - β-thalassemia major
  - Sickle cell anemia
  - Aplastic anemia
  - Severe anemia of any cause
- Hypovolemia due to hemorrhage
- Blood loss during surgery.

Platelet Concentrate

Platelet concentrate may be obtained from a single donor or pooled plasma. Platelets can also be obtained from a single donor by platelet apheresis. They are stored at 20° to 24°C and have shelf-life of 5 days. Platelets have ABO antigens on their surface but do not express Rh antigen. But it is advisable to transfuse Rh negative persons with platelets only from Rh negative persons.

Indications

Bleeding due to:

- Thrombocytopenia, when platelet count is less than 20,000/cu mm.
  - Immune mediated
  - Secondary
    - Chemotherapy induced
    - Due to leukemia
    - Dilutional
- Abnormal platelet function
- Disseminated intravascular coagulation (DIC).

Plasma

Plasma is separated from red cells by centrifugation of the whole blood. The red cells are used as packed red cell component. Plasma may be used to replace the lost plasma proteins in cases of extensive burns.

Fresh Frozen Plasma (FFP)

FFP is plasma (after separation from red blood cells) along with anticoagulant preservative frozen immediately at –2°C. It contains maximum levels of labile and non-labile clotting factors. FFP has a shelf-life of 1 year.

Indications

- Patients on anticoagulant drug therapy (Coumarin)
- Antithrombin deficiency
- Liver diseases

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- Vitamin K deficiency
- Thrombotic thrombocytopenic purpura (TTP)
- Disseminated intravascular coagulation (DIC).

Cryoprecipitate AHF

It is the cold insoluble portion of plasma that precipitates when FFP is thawed (melted) between 1° to 6° C. It contains concentrated precipitate of Factor VIII, XIII, vWF and fibrinogen.

Indication

Hemophilia, factor XIII deficiency, von Willebrand disease and hypofibrinogenemia

Apheresis

It is the method of collection of anticoagulated whole blood from donor, its separation into components, retention of the desired components and returning the remaining constituents to the donor itself. Indications for apheresis are mentioned in Table 60.4.

Table 60.4: Indications for apheresis

- To collect various components for transfusion
  - Platelets—Platelet pheresis
  - Leukocytes
  - Plasma—Plasmapheresis
  - Peripheral stem cells
- Therapeutic apheresis: To remove pathological components like cells, plasma or plasma constituents- plasmapheresis
  - Thrombotic thrombocytopenic purpura
  - Goodpasture syndrome
  - Gullain-Barré syndrome

Granulocyte Transfusion

It is not frequently employed, because it is preferable to administer the growth factors for myelopoiesis like G-CSF/GM-CSF. But it is indicated when granulocyte count is less than 500/cu mm (agranulocytosis), and to combat infections (in neonatal sepsis and chronic granulomatous disease).

TRANSFUSION REACTIONS

Blood transfusion is useful and life saving when performed with caution and with clear indication. Sometimes (about 2-4% of cases) unfavorable complications occur in spite of precaution and preventive measures, which are known as transfusion reactions. They may be broadly divided into infectious and non-infectious complications (Table 60.5).

Noninfectious Complications

Transfusion reactions may result from immune and nonimmune mechanisms.

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Table 60.5: Complications of blood transfusion

- **Noninfectious complications**
  - Immediate reactions
    - Immunological
      - Hemolytic transfusion reactions
      - Febrile nonhemolytic reaction
      - Allergic reaction
      - Anaphylactic
    - Nonimmunological
      - Circulatory overload
      - Air embolism
  - Delayed reactions
    - Immunological
      - Alloimmunization
      - Hemolytic transfusion reactions (mostly asymptomatic)
      - Transfusion associated graft versus host disease
    - Nonimmunological
      - Iron overload-Transfusion hemosiderosis
      - Thrombophlebitis
- **Infectious complications**
  Few of the diseases transmitted by transfusion are:
  - Hepatitis (HBV, HCV and HDV)
  - HIV (AIDS)
  - Malaria
  - Cytomegalovirus
  - Syphilis

Immune Mediated (Immediate and Delayed) Reactions

Acute hemolytic transfusion reaction

- ***ABO incompatibility*** between recipient and donor, resulting in destruction of donor cells. This is brought out by the naturally occurring (preformed) antibodies namely anti-A and anti-B (depending on the blood group).
  - **Massive intravascular hemolysis:** This is one of the serious complications which develop within 1 to 4 hours and with only a few millilitres of incompatible red cells. The preformed IgM antibodies (anti-A and anti-B) in the recipient coat transfused donor red cells and activate complement system to form membrane attack complex (C5-9). This results in intravascular destruction (hemolysis) of transfused donor red blood cells.
    - Patient typically develops fever with chills, pain in the lumbar region, dyspnea, tachycardia and hypotension.
    - Intravascular hemolysis results in hemoglobinuria and oliguria with acute renal failure. Red cell lysis may result in disseminated intravascular coagulation.
    - In majority of cases, this results from clerical errors like wrong labeling of the cross-match sample, improper checking or transfusion to the wrong recipient.
    - Treatment consists of immediate interruption of transfusion, immunosuppression with corticosteroid therapy and management of the renal failure.
- ***Rh incompatibility:*** Because anti-Rh antibodies are not complement-fixing, in Rh incompatibility hemolysis develops in the extravascular compartment.

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Delayed hemolytic transfusion reaction

In the recipient, if antibody titer is too low and weak to be detected during cross-match, it may not cause immediate hemolysis at the time of transfusion. Instead, it may lead to a gradual increase in the antibody titer causing delayed, gradual lysis of donor red cells and is known as a delayed hemolytic transfusion reaction. The features are:

- It is mild and is due to extravascular hemolysis caused by HLA antibodies.
- Develops 4 to 14 days after transfusion.
- Mostly asymptomatic and characterized by unexplained anemia and jaundice.

Febrile nonhemolytic reaction

Patient develops fever, chills, rigors and headache 30 to 60 minutes after transfusion. It is due to sensitization to leukocyte antigens.

Allergic reaction

Allergic reaction manifests as urticaria (hives), fever, bronchospasm and rarely anaphylactic shock. It is due to exposure of allergens in donor's plasma to IgE antibodies in recipient's plasma, which activates mast cells and releases histamine/leukotrienes.

Transfusion associated graft versus host disease (TA-GVHD)

Transfusion associated GVHD is a rare but potentially fatal complication of blood transfusion. This may develop 10 to 12 days after transfusion.

Mechanism: Donor blood products contain mature T cells which attack recipient's tissues. It is observed more commonly in immunocompromised recipients. Clinical features include fever, generalized skin rash, jaundice, diarrhea and features of pancytopenia (due to marrow failure). TA-GVHD can be prevented by transfusing the irradiated blood/components in patients at risk.

Nonimmunological (Immediate and Delayed) Reactions

Circulatory overload

Following whole blood transfusion, the blood volume and venous pressure also increases. This may be significant in the elderly, pregnant women and those with reduced cardiac function resulting in acute pulmonary edema. This can be avoided using blood components like packed red blood cells.

Air embolism

This was common earlier when transfusion was given from the glass bottle. Presently, due to the use of plastic bags with closed tubing system, this complication does not develop.

Iron overload (transfusion siderosis)

- This is seen in patients who receive multiple transfusions over a period of few years (e.g. thalassemia). The excess iron gets deposited in reticuloendothelial cells of spleen, bone marrow, liver, heart and endocrine glands.
- Iron overload can be prevented or reduced by giving iron chelating agents (like Desferal).

Thrombophlebitis

Inflammation of vein may develop in patients with indwelling catheters.

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Infectious Complications

Transfusion of infected blood may transmit few diseases like AIDS, hepatitis (HBV, HCV and HDV), HTLV-I and II, malaria and cytomegalovirus infection. This is prevented by screening the donors for these common and ominous infections.

Exchange Transfusion

When blood is withdrawn from one vein and is replaced with fresh blood from another vein, it is called as an exchange transfusion. It is usually done to remove the red cells (e.g. in HDN) or antibodies (e.g. TTP, HDN).

Indications

- Hematological disorders
  - Neonatal jaundice due to Rh incompatibility (HDN)
  - Thrombotic thrombocytopenic purpura (TTP)
  - Disseminated intravascular coagulation (DIC)
- Nonhematological disorders
  - Goodpasture syndrome
  - Gullain-Barré syndrome

SUMMARY

- Transfusion medicine comprises transfusion of blood and blood components.
- Blood donor should be healthy and the blood is collected in a plastic bag with CPD-A as an anticoagulant.
- Before transfusion of blood or its components, compatibility testing has to be done.
- The various blood components include: packed red blood cells, platelet concentrate, plasma, fresh frozen plasma and cryoprecipitate.
- Transfusion reactions may be infectious and non-infectious. The noninfectious complications may be immediate or delayed and both of which may be brought out by immunological or nonimmunological mechanisms.
- Transfusion may transmit disease like AIDS, hepatitis, malaria and CMV.

SELF-ASSESSMENT EXERCISES

I. Short Notes

1. Anticoagulants used in blood bank
2. Screening of blood donors and enumerate tests used for screening
3. Blood transfusion
4. Compatibility testing

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5. Diseases transmitted by transfusion
6. Transfusion reaction
7. Blood components and their uses

II. Multiple Choice Questions

1. **The function of adenine in the anticoagulant citrate phosphate dextrose for blood collection, in the blood bank is:**
   A. To prevent clotting
   B. To generate glucose
   C. To buffer the end product of glycolysis
   D. To act as a substrate for red cell synthesis of ATP
2. **Platelets are stored at**
   A. 20° to 24°C
   B. –10°C + 2°C
   C. –15°C + 2°C
   D. –37°C + 2°C
3. **The blood product of choice for von Willebrand disease is**
   A. Platelets
   B. Fresh frozen plasma
   C. Whole blood
   D. Cryoprecipitate
4. **The disease NOT transmitted by blood transfusion is**
   A. Gonorrhea
   B. HIV (AIDS)
   C. Malaria
   D. Cytomegalovirus infection

ANSWERS

1. D **2.** A **3.** D **4.** A

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CHAPTER 61 Hematopoietic Stem Cell Transplantation

CHAPTER OUTLINE

- Introduction
- Autologous Stem Cell Transplant
- Allogeneic Stem Cell Transplantation
- Complications of HSC Transplantation

INTRODUCTION

As treatment and possible cure for certain malignant disorders, high-doses of chemotherapy and/or radiotherapy are administered in order to destroy the malignant cells. The dose may exceed the tolerance limit of bone marrow and eliminates or nearly eliminates (ablates) the patient's marrow.

Transfer of organ, tissue or cells is known as transplantation and hematopoietic stem cells (HSC) transplantation is a curative therapy for many hematologic (malignant and nonmalignant) and nonhematologic disorders.

Definition: Hematopoietic stem cell transplantation is a the process of collection and infusion of hematopoietic stem cells obtained from bone marrow (bone marrow transplantation [BMT]) or peripheral blood (blood stem cell transplantation). The source may be either from other individual or own hematopoietic stem cells.

Sources of Hematopoietic Stem Cells

- **Bone marrow:** This is the richest source of hematopoietic stem cells.
- **Peripheral blood:** Few hematopoietic stem cells are present in the circulation, but can be mobilized from bone marrow by administering G-CSF or GM-CSF.
- **Umbilical cord blood:** It is easily available following delivery and is a rich source of hematopoietic stem cells.

Types of Hematopoietic Stem Cell Transplant

- Autologous ("from self"): The patient's own HSCs are removed, cryopreserved and reinfused.
- Allogeneic ("from different genes"): HSCs are obtained from another individual.
- Syngeneic ("from same genes"): HSCs are obtained from an identical twin.

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Indications for HSC transplantation (Table 61.1).

Table 61.1: Indications for hematopoietic stem cell transplantation

Red blood cell disorders
- Aplastic anemia
- Thalassemia major
- Fanconi anemia

WBC disorders
- Leukemia
  - Acute lymphoblastic leukemia –Relapse after initial chemotherapy induced remission
  - Chronic myeloid leukemia
- Myelodysplastic syndromes
- Lymphomas: Hodgkin lymphoma, Non-Hodgkin lymphoma
- Multiple myeloma

Solid tumors, e.g. Carcinoma breast, ovarian cancer, germ cell tumors, neuroblastoma

Immunological disorders
- Severe autoimmune disorders: Scleroderma, Lupus erythematosus
- Immune deficiency syndromes

AUTOLOGOUS STEM CELL TRANSPLANT

Marrow or peripheral stem cells are obtained from the patient before the high-dose therapy, frozen (cryopreserved) and then reinfused after the high-dose therapy to re-constitute marrow function.

Different steps of autologous stem cell transplantation (Fig. 61.1)

- **Harvesting:** During harvesting, bone marrow and/or peripheral HSCs (identified as CD34+ by immunophenotyping) are collected.
- **Processing of HSCs:** The collected bone marrow or peripheral blood stem cells are suspended in dimethyl sulfoxide (prevents ice crystallization in the cells) and frozen in liquid nitrogen. The HSCs can survive in frozen state for at least 5 years.
- **Conditioning:** Before the autologous transplant, the patient is given a high-dose chemotherapy and/or radiation therapy, sometimes total-body irradiation (TBI). This procedure is called conditioning, the purpose of which is to eradicate the recipient's

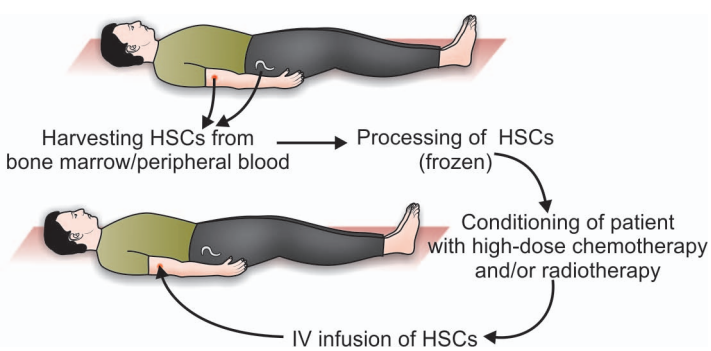


Fig. 61.1: Autologous stem cell transplantation

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hematopoietic and immune system and also malignant tumor cells if any. Conditioning also makes physical space available for HSCs to engraft. In contrast to allogeneic transplantation, autologous transplantation does not require immunosuppression to prevent graft versus host disease (GVHD).

- **Stem cell transplant:** The collected frozen bone marrow or peripheral HSCs are thawed and infused intravenously like a blood transfusion. These stem cells home from the peripheral circulation to the conditioned empty marrow space. Because of conditioning, the patient is pancytopenic and requires critical care. The high-dose chemotherapy also causes breakdown of the normal mucosal barriers in the mouth and gut resulting in increased susceptibility to infections and painful ulcerations.
- **Post-transplant engraftment:** During this stage, the hematopoietic cells produce all the three formed elements of the blood.

ALLOGENEIC STEM CELL TRANSPLANTATION

The HSCs are obtained from an HLA-matched or HLA-mismatched family member (usually a sibling) or an unrelated donor.

Different steps of allogeneic stem cell transplantation (Fig. 61.2)

- **Harvesting:** During harvesting, collected bone marrow and/or peripheral HSCs are mixed with mature WBCs, important being lymphocytes. Mature T lymphocytes are the principal effectors of cell-mediated immunity and both therapeutic benefit and toxicities of allogeneic HSC transplant are due to immunologic reactions between donor T cells and recipient cells.

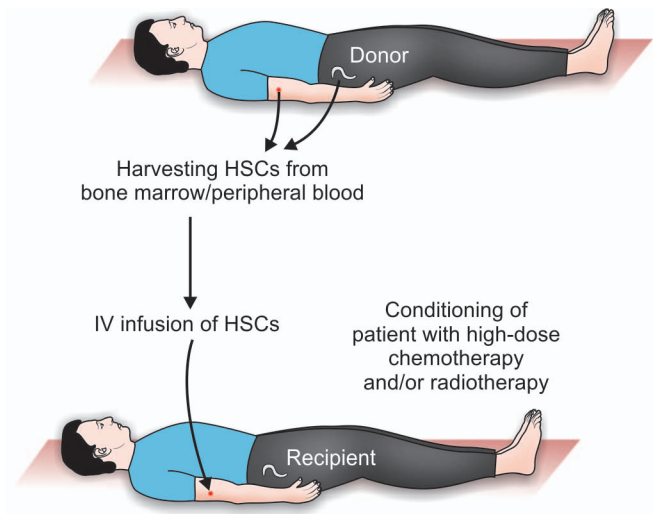


Fig. 61.2: Allogeneic stem cell transplantation

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- **Conditioning:** This step is similar to autologous HSC transplant. Immunosuppression is to allow engraftment of the transplanted HSCs. The patient's endogenous lymphocytes must be suppressed by immunosuppression so as to prevent graft versus host disease (GVHD).
- **Stem cell transplant:** The harvested HSCs are infused into the vein just like a blood transfusion. The stem cells travel and reside in the marrow. Patient requires utmost care during this period similar to autologous transplant.
- **Engraftment:** Donor T lymphocytes help donor HSCs engraft. These T lymphocytes are mainly CD8+ and they destroy any remaining host immune cells which may reject the donor HSCs. Depletion of donor T lymphocytes before allogeneic HSC transplant significantly increases the risk of graft failure. But the greatest disadvantage due to the donor T lymphocytes is development of GVHD.

COMPLICATIONS OF HSC TRANSPLANTATION

Autologous HSC transplants have fewer immunologic complications but have higher rates of relapse of the disease after transplant. Allogeneic HSC transplants have lower rates of relapse but have more immunologic complications, including GVHD, which can be fatal.

- ***Infections:*** Patients are susceptible to a variety of infections (bacterial, viral and fungal) due to lack of granulocytes, as well as lack of a functioning immune system.
- ***Organ toxicity:*** The other complications include damage to GI tract, liver and lungs.
- ***Graft versus host disease:*** GVHD is the major complication that follows allogeneic HSC transplant. This is caused by infused donor T lymphocytes reacting against the recipient's tissues/organs. Three conditions are necessary for the development of GVHD:
  - An immunocompetent graft (i.e. one containing T cells).
  - HLA mismatch (minor or major) between donor and recipient.
  - An immunosuppressed recipient who cannot mount an immune response to the graft.

  If GVHD occurs before 100 days, it is termed as acute GVHD. It often affects three primary target organs simultaneously, namely skin, gastrointestinal (GI) tract and liver. GVHD that occurs after day 100 is termed chronic GVHD and can affect the skin, GI tract, liver, eyes, lungs and joints. GVHD is difficult to treat and in severe cases it is usually fatal.

SUMMARY

- HSC transplantation is used for the treatment of many nonmalignant and malignant disorders.
- The source of HSCs may be bone marrow, peripheral blood or umbilical cord blood.
- The types of transplantation include, autologous ("from self"), allogeneic ("from different genes") or syngeneic (from identical twin).
- A major complication of mainly allogeneic transplant is GVHD.

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SELF-ASSESSMENT EXERCISES

I. Short Note

Hematopoietic stem cell transplantation

II. Multiple Choice Questions

1. **All the following are the sources of hematopoietic stem cells EXCEPT**
   A. Bone marrow
   B. Splenic sinusoid
   C. Peripheral blood
   D. Umbilical cord blood
2. **In autologous hematopoietic stem cell transplantation, the source is**
   A. Patient's own HSCs
   B. HSCs from another individual
   C. HSCs from an identical twin
   D. HSCs from family members of the donor
3. **The major complication of hematopoietic stem cell (HSC) transplantation is**
   A. Hepatitis B
   B. Anaphylactic shock
   C. Sudden cardiac failure
   D. Graft-versus-host disease (GVHD)
4. **Graft-versus-host disease (GVHD) is caused when**
   A. Donor T lymphocytes attack host tissues
   B. Immunosuppressive therapy is increased
   C. Blood is transfused following organ transplantation
   D. Lymphocytes are depleted in the transplant recipient

ANSWERS

1. B **2.** A **3.** D **4.** A

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CHAPTER 62 Gastric Function Tests

CHAPTER OUTLINE

- ❖ Tests for Gastric Acid Secretion
- ❖ Other Tests

TESTS FOR GASTRIC ACID SECRETION

Principle: The tests are based on the principle of measuring **basal acid output** (BAO) and **maximal acid output** (MAO) produced by the stomach under the influence of stimulants. These are then compared with the normal values.

Procedure

- Patient is advised to fast overnight.
- The stomach is intubated.
- Measurement of acid secretion in 2-4 samples collected at an interval of 15 minutes, before stimulation is the **basal acid output**. It is expressed as mEq/hr.
- The stomach is then stimulated by a stimulant to secrete maximal acid and 4 samples are collected again at interval of 15 minutes. This is **maximal acid output** and is expressed as mEq/hr.

Stimulants Used for the Test

The different tests for gastric acid secretion are named depending upon the stimulant used for maximal acid output. Few commonly used stimulants are:

- **Histamine:** It was the first stimulant used for the test and is injected subcutaneously.
- **Pentagastrin:** It is the most commonly used and its action is similar to gastrin.
- **Insulin meal (Hollander test):** The principle of the test is that in hypoglycemic state, the direct vagal action on parietal cells causes acid secretion.
- **Tubeless analysis:** A resin-bound dye called diagnex blue is administered orally. The release of dye by the action of gastric acid and its appearance in the urine indicates the presence of gastric acid. The test can be repeated after giving any stimulants.

Significance: Basal acid output and maximal acid output
Increased in:

- Duodenal ulcer
- Zollinger Ellison syndrome.

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Decreased in:
- Pernicious anemia-atrophic gastritis.
- Achlorhydria (with ulcer in the stomach suggests a gastric malignancy).

OTHER TESTS

Tests for Pepsin

Pepsin inhibitors are used for testing pepsin release from pepsinogen. Pepsin is decreased in atrophic gastritis.

Tests for Mucus

In this, the protein content of mucus is measured which is increased in hypertrophic gastritis.

Test for Intrinsic Factor

Intrinsic factor (IF) is required for absorption of vitamin B_{12} in the small intestine.
Intrinsic factor deficiency causes Pernicious anemia. Schilling test is used for the diagnosis of Pernicious anemia.

Test for Blood

Benzidine test or guaiac test detects blood. The test is positive in hematemesis/bleeding in the gastrointestinal tract or due to trauma of intubation.

Test for Gastrin

Serum gastrin is secreted by G cells present in the antropyloric region and proximal duodenal mucosa. It may be tested by the following tests:

Serum gastrin levels: Radioimmune assay (RIA) is commonly used for estimation of serum gastrin.

Gastrin provocation tests: These tests are used to differentiate between gastric acid hypersecretion and hypergastrinemia.
- **Secretin test:** Secretin is intravenously injected.
- **Calcium infusion test:** In this test calcium gluconate is given intravenously for 3 hours.
  In Zollinger Ellison syndrome, characteristically, serum gastrin levels rise by more than 50% of basal value in both the above mentioned tests.

High levels are found in:
- Atrophic gastritis
- Zollinger Ellison syndrome.

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SUMMARY

- Gastric analysis consists of tests for gastric acid, pepsin, mucus, intrinsic factor, blood and gastrin.
- Gastric acid, both basal acid output and maximal acid output, are mainly increased in duodenal ulcer and Zollinger Ellison syndrome. It is decreased in pernicious anemia which also shows intrinsic factor deficiency.
- High levels of gastrin are found in atrophic gastritis and Zollinger Ellison syndrome.

SELF-ASSESSMENT EXERCISES

I. Short Note

Gastric function tests

II. Multiple Choice Questions

1. **Increased basal acid output and maximal acid output is observed in**
   A. Atrophic gastritis
   B. Gastric carcinoma
   C. Duodenal ulcer
   D. Pernicious anemia
2. **Guaiac test is used to detect**
   A. Proteins in CSF
   B. Bile salts in urine
   C. Blood in the stool
   D. Motility of sperms *in vivo*
3. **High levels of serum gastrin are found in**
   A. Linitis plastica
   B. Chronic peptic ulcer
   C. Zollinger Ellison syndrome
   D. Early gastric carcinoma

ANSWERS

1. C **2.** C **3.** C

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CHAPTER 63 Liver Function Tests and Liver Biopsy

CHAPTER OUTLINE

- ❖ Introduction
- ❖ Liver Function Tests
- ❖ Liver Biopsy

INTRODUCTION

Functions of Liver

- Production and excretion of bile.
- Synthesis of plasma proteins such as albumin, fibrinogen and prothrombin.
- Metabolism of proteins, carbohydrates and lipids.
- Storage of vitamins (A, D and B_{12}) and iron.
- Detoxification of toxic substances such as alcohol and drugs.

Aims of investigation: In patients with suspected liver disease the purpose of investigations are shown in Table 63.1.

Table 63.1: Aims of investigation in patients with liver disease

Aim	*Investigations to be performed*
To detect liver abnormality	Liver function test
To measure the severity and activity of liver damage	Liver function tests and coagulation tests
To determine the site and general nature of structural lesion	Radiological imaging
To identify the specific cause	Specific blood test depending on the nature of disease. Liver biopsy in certain lesions.
To investigate possible complications	Depending on the disease (e.g. in cirrhosis to look for esophageal varices)

LIVER FUNCTION TESTS

Liver function tests (LFTs) are groups of laboratory tests designed to give information about the state of a patient's liver (Table 63.2).

Because of multiple and complex functions of the liver, it is necessary to perform several tests for an accurate diagnosis, to assess the severity of the disease and its prognosis.

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Table 63.2: Liver function tests

A. Tests for excretory functions
- Bilirubin metabolism
  - Serum bilirubin estimation
  - Bilirubin in feces
  - Bilirubin in urine
  - Urobilinogen in urine
- Bromosulphthalein (BSP) excretion
- Bile acids (bile salts)

B. Tests of liver cell injury
- Enzymes that detect hepatocellular injury
  - Transaminases (aminotransferases)
    - Serum aspartate transaminases (AST)
    - Serum alanine transaminases (ALT)
    - AST/ALT ratio
  - Lactate dehyrogenase (LDH)
- Enzymes that detect cholestasis
  - Serum alkaline phosphatase
  - γ-glutamyl transpeptidase
  - 5'-Nucleotidase

C. Tests of metabolism and biosynthesis
- Proteins secreted into the blood
  - Serum proteins: Albumin, globulin and albumin: globulin (A:G) ratio
  - Clotting factors: Prothrombin time
- Hepatocyte metabolism: Serum ammonia
- Lipid and lipoprotein metabolism
- Carbohydrate metabolism

D. Immunologic tests

E. Ancillary diagnostic tests

Alpha-fetoprotein (AFP)

Tests for Excretory Functions

Bilirubin Metabolism

Serum bilirubin estimation

It is not a sensitive test for liver dysfunction and may not reflect the degree of liver damage.

Normal values: Total serum bilirubin in adult is less than 1 mg/dL of which conjugated bilirubin is less than 0.25 mg/dL.

***van den Bergh reaction*:** Bilirubin estimation is based on **van den Bergh diazo reaction** by spectrophotometric method. Diazo reagent consists of diazotised sulfanilic acid which forms a conjugated azo compound with the porphyrin rings of bilirubin.

- **Direct van den Bergh reaction**: Water-soluble conjugated bilirubin gives direct van den Bergh reaction with diazo reagent within one minute.
- **Indirect van den Bergh reaction**: Alcohol-soluble unconjugated bilirubin gives positive result with indirect van den Bergh reaction.

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Causes of hyperbilirubinemia (Table 63.3).

Table 63.3: Causes of hyperbilirubinemia

Predominantly unconjugated hyperbilirubinemia

- Excess production of bilirubin: (e.g. hemolytic anemias, ineffective erythropoiesis)
- Reduced hepatic uptake: (e.g. drugs and some cases of Gilbert's syndrome)
- Impaired bilirubin conjugation:
  - Physiological jaundice of newborn
  - Genetic deficiency of UGT: Crigler–Najjar syndrome type I and II
  - Gilbert syndrome
  - Diffuse hepatocellular disease (e.g. viral or drug-induced hepatitis, cirrhosis)

Predominantly conjugated hyperbilirubinemia

- Excretion defects: Dubin–Johnson syndrome, Rotor syndrome
- Biliary obstruction

Hyperbilirubinemia due increased unconjugated and conjugated bilirubin

- Hepatitis

UGT = Uridine diphosphate-glucuronyl transferase

Bilirubin in feces

Significance: **Absence** of fecal excretion of the pigment is seen **in obstructive jaundice** which gives rise to clay-colored stool.

Bilirubin in urine

Significance: Bilirubinuria is **not seen in normal subjects**. Unconjugated bilirubin is not excreted in the urine (e.g., hemolytic anemia) but conjugated bilirubinuria occurs only when there is raised serum conjugated bilirubin (e.g. obstructive jaundice). Bilirubinuria **appears in** patients of **hepatitis before** the patient becomes **jaundiced.**

It is detected in the laboratory by Fouchet's test, foam test or dipstick method (Chapter 50).

Urobilinogen in urine

Significance: Urobilinogen is **normally excreted** in the urine in traces (1-2 mg/dL). An **increase** in urobilinogen in the urine is found in **hepatocellular disorders** (e.g. alcoholic liver disease, cirrhosis) and **hemolytic anemias**. In obstructive jaundice with complete biliary obstruction, urobilinogen disappears from the urine.

It is estimated by Ehrlich's test or by dipstick method (Chapter 50).

Bromosulphthalein Excretion

Bromosulphthalein (BSP) is a dye that follows the same routes of binding, conjugation and excretion as that of bilirubin. BSP is intravenously injected and venous blood after 45 minutes is tested for percentage of injected dye remaining in the blood. Nowadays, this test is not done due to the availability of enzyme estimations which are better indicators of hepatic dysfunction. BSP excretion test is **valuable only in** the diagnosis of **Dubin-Johnson syndrome.**

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Bile Acids (bile salts)

Primary bile acids (cholic acid and cheno-deoxycholic acid) are formed from cholesterol in the hepatocytes. These bile acids are secreted in the bile. In the gut, they undergo deconjugation by the colonic bacteria and produce secondary bile acids (deoxycholic acid and lithocholic acid). Ninety percent of these bile acids are reabsorbed through **enterohepatic circulation** and reach the liver. About 10% are excreted in the feces.

Significance: **Raised** blood **levels of bile acids produce itching** (pruritus). Serum bile acids are **raised in hepatobiliary diseases with cholestasis**. Their presence in the urine can be detected by Hay's sulfur test (Chapter 50).

Tests of Liver Cell Injury

Estimation of serum enzymes is the most sensitive and useful test for diagnosis of various types of liver (both hepatocellular or cholestatic) injury.

Enzymes that Detect Hepatocellular Injury

Transaminases (aminotransferases)

Hepatocellular damage/necrosis is assessed mainly by estimation of transaminases (Table 63.4).

Table 63.4: Common cause of elevated serum transaminases

Degree	*Serum levels*	*Common causes*
Mild	less than 100 U/L	Chronic hepatitis C, hemochromatosis, fatty liver (steatosis)
Moderate	100-300 U/L	Apart from above, it may be found in alcoholic hepatitis, autoimmune hepatitis, Wilson disease
Severe	More than 300 U/L	Acute viral hepatitis, drug toxicity, toxins

Serum aspartate transaminase (AST)

This was formerly known as glutamic oxaloacetic transaminase (SGOT). It is a **mitochondrial enzyme found in the heart, liver, skeletal muscle and kidney** and is liberated into the blood whenever there is damage to their parenchymal cells. Thus, AST may rise in acute necrosis or ischemia of other organs apart from liver cell injury. Normal serum level is 0.20-0.65 μkat/L (12-38 U/L).

Serum alanine transaminase (ALT)

This was formerly called glutamic pyruvic transaminase or SGPT. It is a **cytosolic enzyme** mainly **found in the liver**. Thus, serum ALT is fairly specific for liver tissue and is of greater value in assessing liver cell injury. Normal serum level is 0.12-0.70 μkat/L (7-41 U/L).

AST/ALT ratio

It is usually of little value except if the ratio is more than 3 (with ALT <300 U/L), is suggestive of **alcoholic** hepatitis.

Significance: Uses of transaminase estimations are:

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- Early diagnosis of viral hepatitis
- Alcoholic liver disease
- Cirrhosis.

Lactate dehydrogenase (LDH)
It occurs in the cytoplasm of all cells, highest concentration being found in liver, heart, skeletal muscle, kidney and RBCs. Thus, it is not specific for liver diseases.

Significance
- Marked increase in metastatic carcinoma to the liver.
- Moderate increase in cirrhosis, obstructive jaundice and acute viral hepatitis.

Choline esterase
It is synthesized in the liver and is decreased in hepatocellular disease and malnutrition due to impaired synthesis.

Enzymes that Detect Cholestasis

Serum alkaline phosphatase
It is produced by many tissues (bone, liver, intestine and placenta) and is excreted in the bile. Normal serum alkaline phosphatase (range in adults 33-96 U/L) is mainly derived from bone.

Significance
- **Increased** levels can be found in **diseases of bone, liver and in pregnancy**.
- In the absence of bone disease and pregnancy, an **elevated** serum alkaline phosphatase level (3 to 10 times normal) indicates **biliary tract obstruction**.
- Mild to moderate increase is observed in parenchymal liver diseases (hepatitis, cirrhosis and with hepatic metastasis).

A combination of serum transaminases and alkaline phosphatase is very useful in the diagnosis of injury to liver cells.

γ-glutamyl transpeptidase (γ-GT)
Liver is the primary source of the serum enzyme, γ-GT. Its serum level parallels serum alkaline phosphatase. So it is used to confirm that raised serum alkaline phosphatase is due to **hepatobiliary disease**. It is also raised in patients with **alcohol abuse** even without liver disease.

5′-Nucleotidase
It is also a phosphatase derived from the liver. It is useful to distinguish alkaline phosphatase of hepatic origin from that of bone.

Tests of Metabolism and Biosynthesis

Liver is the main site of synthesis of amino acids and plasma proteins, lipids and lipoproteins, carbohydrates and vitamins.

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Plasma Protein Synthesis

Hepatocytes synthesize several proteins including albumin, fibrinogen, prothrombin, alpha-l-antitrypsin and acute phase reactant proteins. Levels of these plasma proteins are reduced in extensive liver damage. Estimation of the following will be useful in the diagnosis of liver disorders:

Serum proteins

Routinely done estimations are:

- **Total** serum **proteins** (normal 6.7 to 8.6 gm/dL)
- **Serum albumin** (normal 3.5 to 5.5 gm/dL) is produced solely by the liver.
- **Serum globulin** (normal 2 to 3.5 gm/dL)
- **Albumin/globulin** (A/G) **ratio** (normal 1.5-3:1).

They are evaluated by serum/immuno electrophoresis. Previously, thymol turbidity and flocculation tests were used for their estimation.

Significance

Hypoalbuminemia develops with chronic inflammatory disorders such as **cirrhosis** and **chronic hepatitis.**

Clotting factors

Both prothrombin time and partial thromboplastin time, reflect the activities of various clotting factors. They are prolonged in hepatocellular disease.

Prothrombin time

It depends on hepatic synthesis of clotting factors and intestinal uptake of vitamin K (fat- soluble vitamin).

- **Prolonged prothrombin time: Obstruction of the bile duct** and intra-hepatic cholestasis, which result in vitamin K deficiency due to impaired lipid absorption, are associated with prolonged prothrombin time. With parenteral administration of vitamin K, prothrombin time will return to normal, if the prolongation is due to obstruction. If there is no improvement in prothrombin time, it indicates severe hepatocellular disease.

Serum Ammonia

Ammonia can be metabolized only in the liver and is derived mainly from amino acid (received from the diet and tissue breakdown) and nucleic acid metabolism. Ammonia is a toxic substance ultimately converted into nontoxic urea which is readily excreted. **Increased blood level of ammonia** is found when most of the liver is destroyed as in **acute fulminant hepatitis, cirrhosis** and **hepatic encephalopathy**. The elevated levels are due to inability of severely damaged liver to convert ammonia to urea.

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Serum Gamma Globulins (immunoglobulins)

Increased in most forms of **chronic liver disease.**

- IgA is raised in cirrhosis.
- IgG is markedly raised in chronic active hepatitis.
- IgM is markedly increased in primary biliary cirrhosis.

Lipid and Lipoprotein Metabolism

Liver synthesizes cholesterol and cholesterol esters, phospholipids and triglycerides. These lipids are insoluble in water and are carried in circulation as high density lipoproteins (HDL), low density lipoproteins (LDL) and very low density lipoproteins (VLDL).

Total serum cholesterol (normal <200 mg/dL)

- Raised in cholestasis, probably due to retention of cholesterol which is normally excreted in the bile.
- Decreased in acute and chronic diffuse liver diseases and in malnutrition.

Carbohydrate Metabolism

Liver also plays a central role in carbohydrate metabolism. Blood glucose level is reduced in fulminant hepatitis. Impaired glucose tolerance and relative insulin resistance is found in chronic liver disease.

Immunologic Tests

Diseases of liver may be associated with various immunological abnormalities.

Nonspecific Immunologic Reactions

- Smooth muscle antibody to actin component of muscle: It is found in few hepatic disorders (autoimmune hepatitis) with hepatic necrosis.
- Mitochondrial antibody: It is associated with primary biliary cirrhosis.
- Antinuclear antibody: It is found in few patients of chronic hepatitis (autoimmune hepatitis) and LE cell test may be positive in such patients.

Specific Antigens/Antibodies

- **Hepatitis B surface antigen (HBsAg**), **Hepatitis B core antibody (anti-HBc), Hepatitis Be antigen (HBeAg**) in hepatitis B infection.
- Antibodies to entamoeba histolytica can be demonstrated in amoebic liver abscess.

Ancillary Diagnostic Tests

Alpha Fetoprotein

It is an α-globulin found in fetal blood. It originates in fetal liver, GI tract and yolk sac**.** It is one of the tumor markers for hepatocellular carcinoma, but not a specific marker, as it may be found in non-neoplastic and other neoplastic conditions.

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Ultrasonography

Indications

- **Cholestasis:** To visualize the dilated intra- and extrahepatic canalicular tree.
- **Space-occupying lesions (SOLs) within the liver**: To differentiate neoplasms from non-neoplastic cysts.
- **For US-guided FNAC or liver biopsy**.

FNAC and/or Liver Biopsy

Summary of important liver function tests is given in Table 63.5.

Table 63.5: Important liver function tests

Category of test	*Serum measurement*
Liver cell damage	Serum aspartate aminotransferase (AST) Serum alanine aminotransferase (ALT)
Biliary excretion/obstruction	
• Substances normally secreted in bile	Serum bilirubin ▪ Total: Unconjugated plus conjugated ▪ Direct: Conjugated only
• Plasma membrane enzymes released from damaged bile canaliculus	Serum alkaline phosphatase, serum γ-glutamyl transpeptidase
Hepatocyte synthetic function-secretion of proteins into the blood	Serum albumin Prothrombin time (factors V, VII, X, prothrombin, fibrinogen)
Hepatocyte metabolism	Serum ammonia

Reference range of liver function tests are shown in Table 63.6.

Table 63.6: Reference range of liver function tests

Name of the test	*Normal range*
Serum bilirubin	Total bilirubin less than 1 mg/dL Conjugated bilirubin less than 0.25 mg/dL
Serum enzymes	
• Serum aspartate transaminase or AST	0.20-0.65 µkat/L (12-38 U/L)
• Serum alanine transaminase or ALT	0.12-0.70 µkat/L (7-41 U/L)
• Serum alkaline phosphatase	33-96 U/L
Serum proteins	
• Total serum proteins	6.7 to 8.6 gm/dL
• Serum albumin	3.5 to 5.5 gm/dL
• Serum globulin	2 to 3.5 gm/dL
• Albumin/globulin (A/G) ratio	1.5-3:1
Total serum cholesterol	<200 mg/dL

LIVER BIOPSY

Percutaneous needle biopsy of the liver through an intercostal approach is done under local anesthesia. It is a simple, safe procedure for assessment of liver histology for the diagnosis of various liver disorders.

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Before the Biopsy

Prothrombin time (PT) should be performed before liver biopsy. If it is high, patient should be given parenteral vitamin K and PT should be repeated. Liver biopsy is contraindicated if PT is prolonged.

Needle

Vim Silverman's needle (Fig. 63.1) is used for liver biopsy and its different parts are:

- Prongs-split needle (with sharp edges)
- Stilette
- Cannula

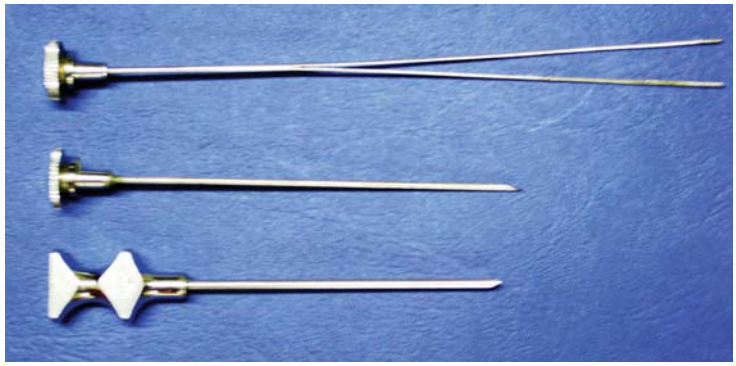


Fig. 63.1: Vim Silverman's needle showing prongs-split needle, stilette and cannula (top to bottom)

Procedure

- Biopsy is performed through the 9th or 10th right intercostal space in the mid-axillary line. The skin is cleaned with iodine followed by alcohol.
- The patient is asked to hold his/her breath and the Vim Silverman's needle with stilette is introduced into the liver through the skin.
- The stilette is removed. The prongs-split needle is introduced into the liver parenchyma and rotated.
- The needle (cannula) along with split needle inside is withdrawn.

Indications

- Unexplained hepatomegaly, splenomegaly, jaundice.
- Cirrhosis of liver: To assess the type, etiology and activity of the disease process.
- Chronic hepatitis: For evaluation of the course.

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- Pyrexia of unknown origin (PUO): May be helpful in the diagnosis of miliary tuberculosis, sarcoidosis or malignancy.
- Idiopathic hemochromatosis: To determine iron stores in liver.

Contraindications

- Congenital coagulation disorders, e.g. hemophilia A and B.
- Prolonged PT.
- Obstructive jaundice: Liver biopsy may cause leakage of bile from liver into the peritoneum causing severe and fatal bile peritonitis.
- Massive ascites
- Patients with severe cough where diaphragm movements cannot be controlled.
- Uncooperative patients
- Hydatid cyst liver
- Hemangioma liver

} These are relative contradictions

Complications

- Hemorrhage from the biopsy site
- Bile peritonitis in patients with obstructive jaundice
- Referred pain to shoulder from right pleura.

SUMMARY

- Liver has multiple and complex functions and single test alone is not useful for **assessing** the liver function. It is necessary to perform several tests for diagnosis and for assessing prognosis.
- Most commonly done tests are estimation of serum enzymes (AST, ALT and alkaline phosphatase), bilirubin (both direct and total), albumin, globulin and their ratio (A: G ratio) and prothrombin time.
- For differentiation of different types of hepatitis, immunological tests are very useful.
- Biopsy of the liver is a simple, safe procedure for assessment of liver histology for the diagnosis of various liver disorders.It should be performed only when prothrombin time is normal. It is indicated in patients with unexplained hepatomegaly, splenomegaly, jaundice, cirrhosis, chronic hepatitis, PUO and idiopathic hemochromatosis.

SELF-ASSESSMENT EXERCISES

I. Short Notes

1. Liver function tests and its importance
2. Indications for liver biopsy
3. Contraindications for liver biopsy

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II. Multiple Choice Questions

1. **The disorder which causes predominantly elevated serum unconjugated bilirubin is**
   A. Viral hepatitis
   B. Hemolytic anemia
   C. Obstructive jaundice
   D. Dubin-Johnson syndrome
2. **If the ratio between AST and ALT is more than 3, it is suggestive of**
   A. Hepatitis B
   B. Yellow fever
   C. Biliary cirrhosis
   D. Alcoholic hepatitis
3. **Marked elevation of serum alkaline phosphatase levels indicates**
   A. Alkalosis
   B. Renal failure
   C. Biliary tract obstruction
   D. Hepatic encephalopathy
4. **The enzymes which is raised in patients with alcohol abuse even in the absence of liver disease is**
   A. Serum alanine transaminase
   B. Serum alkaline phosphatase
   C. γ-glutamyl transpeptidase
   D. Serum aspartate transaminase
5. **Return to normal of a prolonged prothrombin time, after parenteral administration of vitamin K indicates**
   A. Autoimmune hepatitis
   B. Biliary tract obstruction
   C. Massive necrosis of liver cells
   D. Alcoholic hepatitis
6. **The laboratory test that is mandatory before liver biopsy is**
   A. Urine examination
   B. Prothrombin time (PT)
   C. Peripheral smear examination
   D. Blood grouping and cross-matching
7. **One of the indication for liver biopsy is**
   A. Cirrhosis
   B. Obstructive jaundice
   C. Fulminant hepatitis
   D. Prolonged prothrombin time

ANSWERS

1. B **2.** D **3.** C **4.** C **5.** B **6.** B **7.** A

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CHAPTER 64 Renal Function Tests

CHAPTER OUTLINE

- ❖ Tests for Renal Function
- ❖ Renal Biopsy

Functions of Kidney: Kidneys perform various functions.
- Excretion of waste products
- Regulation of acid-base balance
- Regulation of salt and water balance
- Secretion of renin (regulates blood pressure) and erythropoietin (involved in erythropoiesis).

TESTS FOR RENAL FUNCTION

- Urine analysis (both routine and microscopic)
- Blood chemistry
- Renal clearance tests
- Concentration and dilution tests.

Urinalysis is discussed in detail in Chapter 50.

Blood Chemistry

Impaired renal function causes elevation of end-products of protein metabolism which is known as **azotemia.** These include:
- Blood urea (normal range 20 to 40 mg/dL)
- Blood urea nitrogen (BUN): Normal range 10 to 20 mg/dL
- Creatinine (normal range 0.6 to 1.2 mg/dL).

Renal Clearance Tests

These tests measure the **glomerular function** and renal blood flow.
- Glomerular filtration rate (GFR) is the rate at which fluid passes into the nephron after filtration and it measures renal excretory function. The rate of glomerular filtration is measured by the capacity of the kidneys to clear waste products or foreign materials (inulin, mannitol, creatinine and urea) from the blood into the urine.
- Clearance of a solute from plasma into the urine equals GFR, if the solute is freely filtered through the glomerulus and neither secreted into nor absorbed from the renal tubules.

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- From the determination of blood concentration of the test material and the quantity eliminated in the urine, "clearance" can be calculated and is expressed in terms of milliliters of blood cleared per unit time.
  The various tests are:

Inulin Clearance Test

Inulin is ideal for the measurement of GFR because it is neither secreted nor reabsorbed by the tubule. Mannitol can also be used.

Creatinine Clearance

Creatinine is normally released into the plasma by muscle metabolism and is filtered through the glomerulus. Normally, the clearance of endogenous creatinine approximates the glomerular filtration rate. It is easy and routinely employed method for GFR estimation.

Urea Clearance Test

Urea clearance test is less sensitive test.

Concentration/Dilution Tests

These tests are used to evaluate the tubular function. The ability of the nephron to concentrate or dilute urine is dependent on the tubular cells in the renal medulla as well as antidiuretic hormone (ADH).

Urine Specific Gravity

Urine specific gravity (normal range 1.003 to 1.035) is a measure of capacity of the tubules to reabsorb water, thus concentrating the urine (Chapter 50). In chronic tubular damage it remains fixed at 1.010. If the patient's urine contains no sugar or protein and has a specific gravity of 1.025 or higher, a concentration test is unnecessary.

Concentration Test

In this test, an artificial deprivation of fluid is induced in a patient for more than 20 hours. With normal tubular function, water is selectively absorbed and urine will have high solute concentration with specific gravity of 1025 or more. With tubular damage, the specific gravity remains constant irrespective of water deprivation.

Dilution Test

In this test, excessive fluid is given to the patient which will result in a lower solute concentration in a normal individual. The resulting specific gravity will be 1.003 or less. In tubular damage, the specific gravity remains constant irrespective of excessive water intake.

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RENAL BIOPSY

It is used to establish the nature and extent of renal disease. Biopsy is performed transcutaneously with ultrasound guidance. Biopsy material is examined under light microscopy, electron microscopy and by immunofluorescence. Indications, contraindications and complications of renal biopsy are presented in Table 64.1.

Table 64.1: Indications, contraindications and complications of renal biopsy

Indications	*Contraindications*	*Complications*
Chronic renal failure with normal sized kidney	Coagulation disorders	Pain
Unexplained acute renal failure	Thrombocytopenia	Bleeding into the urine
Nephrotic syndrome/glomerular proteinuria in adults	Uncontrolled hypertension	Bleeding around kidney
Nephrotic syndrome in children with atypical presentation/not responding to treatment	Solitary kidney	Arteriovenous fistula
Isolated hematuria/proteinuria	Kidney less than 60% predicted size	

SUMMARY

- Renal function can be assessed by urine analysis (both routine and microscopic), blood chemistry, renal clearance tests, and concentration and dilution tests.
- Impaired renal function results in raised values of end-products of protein and can be assessed by estimation of blood urea, blood urea nitrogen and serum creatinine.
- Renal clearance tests are used to assess the glomerular function whereas concentration and dilution tests are for tubular function.
- Renal biopsy is useful for establishing the nature and extent of renal disease.

SELF-ASSESSMENT EXERCISES

I. Short Note

Renal function tests

II. Multiple Choice Questions

1. **Normal range for blood urea is**
   A. 10 to 20 mg/dL
   B. 20 to 40 mg/dL
   C. 40 to 60 mg/dL
   D. 60 to 80 mg/dL

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2. The ideal test which measures the glomerular function and renal blood flow is

A. Dilution test
B. Concentration test
C. Inulin clearance test
D. Urine specific gravity

ANSWERS

1. B **2.** C

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CHAPTER 65 Thyroid Function Tests

CHAPTER OUTLINE

- ❖ Introduction
- ❖ Thyroid Function Tests

INTRODUCTION

Thyroid function tests (TFTs) is a collective term for test used to check the function of the thyroid gland.

Indications

Screening for Thyroid Dysfunction

Thyroid function tests are performed to screen patients suspected of having thyroid disease. The indications for screening include:

- Neonates for congenital hypothyroidism
- Patients with autoimmune disease
- Individuals with strong family history of thyroid diseases
- Patient with suspected hyperthyroidism/hypothyroidism
- Conditions linked to thyroid disease such as atrial fibrillation or anxiety disorder.

Surveillance

- Women with a history of postpartum thyroiditis
- Post neck irradiation
- Post destructive treatment (radioiodine or surgical) for thyrotoxicosis.

Monitoring

One of the common reasons for assessing thyroid function is to monitor therapy.

- Monitoring treatment of **hyperthyroidism** with antithyroid drugs.
- Patients with primary **hypothyroidism** who are taking thyroxine hormone replacement therapy.

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THYROID FUNCTION TESTS (TABLE 65.1)

Serum Thyroid Hormones

Thyroid Stimulating Hormone/thyrotrophin (TSH)

It is secreted by the anterior pituitary gland. It increases production and release of thyroxine (T_3 and T_4) form the thyroid. It secretion is inhibited by high levels of T_3 and T_4. It is stimulated by high levels of TRH and low levels of T_3 and T_4.

Table 65.1: Thyroid function tests

Serum Thyroid Hormones
- Thyroid stimulating hormone/thyrotrophin (TSH)
- Thyroid hormone levels
  - Free Thyroxine (FT_4)
  - Triiodothyronine (T_3)
- Thyrotrophin releasing hormone (TRH) stimulation test
- Thyroglobulin (Tg)
- Calcitonin level

Thyroid Autoantibody Tests
- Anti-microsomal antibody
- Anti-thyroid peroxidase antibody (TPOAb)
- Anti-thyroglobulin antibody (TgAb)
- TSH receptor antibody (TRAb)

Radioactive Iodine Uptake (RAIU)

Use: To assess true metabolic status
- **Increased:** Thyroid failure is characterized by decrease in the level of T_3 and T_4 with increased TSH.
  - Primary hypothyroidism
  - Hashimoto thyroiditis.
- **Decreased:** In hyperthyroidism TSH level is suppressed and may be undetectable.

Thyroid Hormone Levels

- **FT_4 (Free Thyroxine):** It is produced only by the thyroid gland. It travels in plasma bound to protein or in free form. It is converted to T_3 (active) and RT_3 (inactive) peripherally in blood and tissues.
- **T_3 (Triiodothyronine):** T_4 is converted to T_3 in the peripheral tissue. Most of it is transported in plasma bound to protein and only 0.3% in free form.

  ***Use*:** Its level reflects secretory activity.
  - **Increased in hyperthyroidism**
  - **Decreased in hypothyroidism**

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Thyrotrophin Releasing Hormone (TRH) Stimulation Test

TRH Secreted by hypothalamus, which stimulates the release of TSH from the pituitary. TRH secretion is inhibited by high levels of T_3 and T_4 and stimulated by low levels of T_3 and T_4. Serum TSH is measured before and after the intravenous administration of TRH.

Use: To differentiate two forms (whether due to tumor or not) of thyrotrophin induced hyperthyroidism.

- In primary hypothyroidism an exaggerated prolonged rise of already increased TSH level is observed.
- In secondary hyperthyroidism (pituitary), there will be no rise in the decreased TSH level.

Thyroglobulin (Tg)

It is secreted by thyroid follicular cells and is involved in iodination and synthesis of thyroid hormones.

Use: To predict the outcome of therapy for hyperthyroidism.

- **Increased**
  - Well differentiated thyroid carcinoma
  - Hyperthyroidism
- **Decreased:** Total thyroidectomy or destruction of thyroid by radiation.

Calcitonin Level

It is secreted by the parafollicular C cells of the thyroid. It decreases the bone-resorption by osteoclasts and causes decreased serum calcium.

Use: Diagnosis of primary/recurrence of medullary carcinoma of thyroid or metastases after the removal of the primary.

- **Increased in:**
  - Some paraneoplastic syndromes (e.g. carcinoma of lung)
  - Hypercalcemia of any cause
  - C cell hyperplasia.

Thyroid Autoantibody Tests

The different types of thyroid autoantibodies responsible for the autoimmune thyroid disorders are:

- Anti-microsomal antibody
- Anti-thyroid peroxidase (TPO) antibody (TPOAb): They are involved in the tissue destructive process associated with hypothyroidism in Hashimoto and atrophic thyroiditis.
- Anti-thyroglobulin (Tg) antibody
- TSH receptor (TR) antibody (TRAb): These antibodies were previously known as thyroid stimulating immunoglobulin (TSI) or long-acting thyroid stimulators (LATS).
  Laboratory assessment of antithyroid peroxidase antibodies is replacing the anti-microsomal and anti-thyroglobulin antibodies.

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Use

- Hashimoto disease
- Graves disease.

Radioactive Iodine Uptake (RAIU)

T_4 contains high iodine and the thyroid gland must take-up a large amount of iodine from the circulating blood for the synthesis of T_4. The iodine uptake activity of thyroid can be measured by administering orally a low dose of radioactive iodine (or technetium) and the radioactivity over the thyroid is measured at specified intervals. The amount of radioactivity that is taken up by the thyroid gland is known as radioactive iodine uptake (RAIU). Normal uptake ranges from 10 to 35% in 24 hours. A very high RAIU is seen in hyperthyroidism, while a low RAIU is seen hypothyroidism.

Uses

- Evaluation of hyperthyroidism
- Differentiate Graves from toxic goiter
- Function of a thyroid nodule as hot or cold.

- **Increased:** Graves disease, toxic multinodular goiter and adenoma and early thyroiditis.
- **Decreased:** Hypothyroidism, late thyroiditis.

Contraindication: RAIU is contraindicated in children and during pregnancy/lactation.

Thyroid Scan

In addition to the radioactive iodine uptake, a thyroid scan may be obtained, which shows a picture of the thyroid gland

Common thyroid function tests in normal and pathological conditions is shown in Table 65.2.

Table 65.2: Common thyroid function tests in normal and pathological states

Thyroid functional state	*TSH*	*Free T_4*	*T_3*	*Tg*
Euthyroid	Normal	Normal	Normal	Normal
Hyperthyroidism-Graves' disease	Decreased or undetectable	Increased	Increased	Increased
Hypothyroidism	Increased	Decreased	Decreased	Normal or decreased
Hashimoto thyroiditis hypothyroidism	High	Normal or decreased	Normal or decreased	Normal or decreased

Normal reference range for common thyroid functions are presented in Table 65.3.

Table 65.3: Normal range for thyroid function tests

Test	*Normal range*
Thyroid Stimulating Hormone (TSH)	0.4-4.5 µIU/mL
T_3-total	60-181 ng/mL
T_4-free	0.8-1.5 ng/dL
T_4-total	5.5-12.3 ng/mL
Radioactive iodine uptake (RAIU)	10 to 35% in 24 hours

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SUMMARY

- Thyroid function tests are useful for evaluation of hyper or hypothyroidism. It is also useful for diagnosis of autoimmune diseases of thyroid.
- Estimation of serum calcitonin is of value in diagnosis of primary or recurrence in medullary carcinoma.

SELF-ASSESSMENT EXERCISES

I. Short Note

Thyroid function tests

II. Multiple Choice Questions

1. **Thyrotrophin (TSH) is increased in**
   A. Hyperthyroidism
   B. Hashimoto thyroiditis
   C. Follicular adenoma
   D. Papillary carcinoma of thyroid
2. **Estimation of the following is useful for the diagnosis of primary/recurrence of medullary carcinoma of thyroid**
   A. TRH
   B. TSH
   C. Calcitonin
   D. Thyroglobulin

ANSWERS

1. B **2.** C

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Appendix

WHO CLASSIFICATION OF TUMORS OF HEMATOPOIETIC AND LYMPHOID TISSUES

A. MYELOPROLIFERATIVE NEOPLASMS

Chronic myelogenous leukemia, *BCR-ABL1* positive
Chronic neutrophilic leukemia
Polycythemia vera
Primary myelofibrosis
Essential thrombocythemia
Chronic eosinophilic leukemia, NOS
Mastocytosis
- Cutaneous mastocytosis
- Systemic mastocytosis
- Mast cell leukemia
- Mast cell sarcoma
- Extracutaneous mastocytoma

Myeloproliferative neoplasm, unclassifiable

B. MYELOID AND LYMPHOID NEOPLASMS WITH EOSINOPHILIA AND ABNORMALITIES OF *PDGFRA, PDGFRB OR FGFR1*

Myeloid and lymphoid neoplasms with *PDGFRA* rearrangement
Myeloid and lymphoid neoplasms with *PDGFRB* rearrangement
Myeloid and lymphoid neoplasms with *FGRFR1* abnormalities

C. MYELODYSPLASTIC/MYELOPROLIFERATIVE NEOPLASMS

Chronic myelomonocytic leukemia
Atypical chronic myeloid leukemia, *BCR-ABL1* negative
Juvenile myelomonocytic leukemia
Myelodysplastic/myeloproliferative neoplasm, unclassifiable
Refractory anemia with ring sideroblasts associated with marked thrombocytosis

D. MYELODYSPLASTIC SYNDROMES

Refractory cytopenia with unilineage dysplasia
- Refractory anemia
- Refractory neutropenia
- Refractory thrombocytopenia

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Refractory anemia with ring sideroblasts
Refractory cytopenia with multilineage dysplasia
Refractory anemia with excess blasts
Myelodysplastic syndrome with isolated del(5q)
Myelodysplastic syndrome, unclassifiable
Childhood myelodysplastic syndrome
Refractory cytopenia of childhood

E. ACUTE MYELOID LEUKEMIA (AML) AND RELATED PRECURSOR NEOPLASMS

AML with recurrent genetic abnormalities
AML with t(8;21)(q22;q22); *RUNX1-RUNX1T1*
AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22); *CBFB-MYH11*
Acute promyelocytic leukemia with t(15;17)(q22;q12); *PML-RARA*
AML with t(9;11)(p22;q23); *MLLT3-MLL*
AML with t(6;9)(p23;q34); *DEK-NUP214*
AML with inv(3)(q21q26.2) or t(3;3)(q21;q26.2); *RPN1-EVI1*
AML (megakaryoblastic) with t(1;22)(p13;q13); *RBM15-MKL* 1
AML with mutated NPM1
AMLwith mutated CEBPA
AML with myelodysplasia-related changes
Therapy-related myeloid neoplasms
Acute myeloid leukemia, NOS
AML with minimal differentiation
AML without maturation
AML with maturation
Acute myelomonocytic leukemia
Acute monoblastic and monocytic leukemia
Acute erythroid leukemia
Acute megakaryoblastic leukemia
Acute basophilic leukemia
Acute panmyelosis with myelofibrosis
Myeloid sarcoma
Myeloid proliferations related to Down syndrome
Transient abnormal myelopoiesis
Myeloid leukemia associated with Down syndrome
Blastic plasmacytoid dendritic cell neoplasm

F. ACUTE LEUKEMIAS OF AMBIGUOUS LINEAGE

Acute undifferentiated leukemia
Mixed phenotype acute leukemia with t(9;22)(q34;q11.2); *BCR-ABL1*
Mixed phenotype acute leukemia with t(v;11q23); *MLL* rearranged

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Mixed phenotype acute leukemia, B/myeloid, NOS
Mixed phenotype acute leukemia, T/myeloid, NOS
Natural killer (NK) cell lymphoblastic leukemia/lymphoma

G. PRECURSOR LYMPHOID NEOPLASMS

B lymphoblastic leukemia/lymphoma
B lymphoblastic leukemia/lymphoma, NOS
B lymphoblastic leukemia/lymphoma with recurrent genetic abnormalities
B lymphoblastic leukemia/lymphoma with t(9:22)(q34;q11.2); *BCR-ABL 1*
B lymphoblastic leukemia/lymphoma with t(v;11q23); *MLL* rearranged
B lymphoblastic leukemia/lymphoma with t(12;21)(p13;q22); *TEL-AML1 (ETV6-RUNX1)*
B lymphoblastic leukemia/lymphoma with hyperdiploidy
B lymphoblastic leukemia/lymphoma with hypodiploidy (hypodiploid ALL)
B lymphoblastic leukemia/lymphoma with t(5;14)(q31;q32); *IL3-IGH*
B lymphoblastic leukemia/lymphoma with t(1;19)(q23;p13.3); *E2A-PBX1(TCF3-PBX1)*
T lymphoblastic leukemia/lymphoma

H. MATURE B-CELL NEOPLASMS

Chronic lymphocytic leukemia/small lymphocytic lymphoma
B cell prolymphocytic leukemia
Splenic B cell marginal zone lymphoma
Hairy cell leukemia
Splenic B cell lymphoma/leukemia, unclassifiable
- *Splenic diffuse red pulp small B cell lymphoma*
- *Hairy cell leukemia-variant*

Lymphoplasmacytic lymphoma
- Waldenström macroglobulinemia

Heavy chain diseases
- Alpha heavy chain disease
- Gamma heavy chain disease
- Mu heavy chain disease

Plasma cell myeloma
Solitary plasmacytoma of bone
Extraosseous plasmacytoma
Extranodal marginal zone lymphoma of mucosa-associated lymphoid tissue (MALT lymphoma)
Nodal marginal zone lymphoma
- *Pediatric nodal marginal zone lymphoma*

Follicular lymphoma
- *Pediatric follicular lymphoma*

Primary cutaneous follicle center lymphoma
Mantle cell lymphoma
Diffuse large B cell lymphoma (DLBCL), NOS

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- T cell/histiocyte rich large B-cell lymphoma
- Primary DLBCL of the CNS
- Primary cutaneous DLBCL, leg type
- *EBV positive DLBCL of the elderly*

DLBCL associated with chronic inflammation
Lymphomatoid granulomatosis
Primary mediastinal (thymic) large B cell lymphoma
Intravascular large B cell lymphoma
ALK positive large B cell lymphoma
Plasmablastic lymphoma
Large B cell lymphoma arising in HHV8-associated multicentric Castleman disease
Primary effusion lymphoma
Burkitt lymphoma
B cell lymphoma, unclassifiable, with features intermediate between DLBCL and Burkitt lymphoma
B cell lymphoma, unclassifiable, with features intermediate between DLBCL and classical Hodgkin lymphoma

I. MATURE T CELL AND NK CELL NEOPLASMS

T cell prolymphocytic leukemia
T cell large granular lymphocytic leukemia
Chronic lymphoproliferative disorder of NK cells
Aggressive NK cell leukemia
Systemic Epstein-Barr virus (EBV) positive T cell lymphoproliferative diseases of childhood
Hydroa vacciniforme-like lymphoma
Adult T cell leukemia/lymphoma
Extranodal NK/T cell lymphoma, nasal type
Enteropathy-associated T cell lymphoma
Hepatosplenic T cell lymphoma
Subcutaneous panniculitis-like T cell lymphoma
Mycosis fungoides
Sezary syndrome
Primary cutaneous CD30 positive T cell lymphoproliferative disorders

- Lymphomatoid papulosis
- Primary cutaneous anaplastic large cell lymphoma

Primary cutaneous gamma-delta T cell lymphoma
Primary cutaneous CD8 positive aggressive epidermotropic cytotoxic T cell lymphoma
Primary cutaneous CD4 positive small/medium T cell lymphoma
Peripheral T cell lymphoma, NOS
Angioimmunoblastic T cell lymphoma
Anaplastic large cell lymphoma, ALK positive
Anaplastic large cell lymphoma, ALK negative

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J. HODGKIN LYMPHOMA

Nodular lymphocyte predominant Hodgkin lymphoma
Classical Hodgkin lymphoma
- Nodular sclerosis classical Hodgkin lymphoma
- Lymphocyte-rich classical Hodgkin lymphoma
- Mixed cellularity classical Hodgkin lymphoma
- Lymphocyte-depleted classical Hodgkin lymphoma

K. HISTIOCYTIC AND DENDRITIC CELL NEOPLASMS

Histiocytic sarcoma
Langerhans cell histiocytosis
Langerhans cell sarcoma
Interdigitating dendritic cell sarcoma
Follicular dendritic cell sarcoma
Fibroblastic reticular cell tumor
Intermediate dendritic cell tumor
Disseminated juvenile xanthogranuloma

L. POST-TRANSPLANT LYMPHOPROLIFERATIVE DISORDERS (PTLD)

Early lesions
- Plasmacytic hyperplasia
- Infectious mononucleosis-like PTLD

Polymorphic PTLD
Monomorphic PTLD (B- and T/NK-cell types)
Classical Hodgkin lymphoma type PTLD

NOS, not otherwise specified

The italicised histologic types are provisional entities, for which the WHO Working Group felt there was insufficient evidence to recognize as distinct diseases at this time.

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