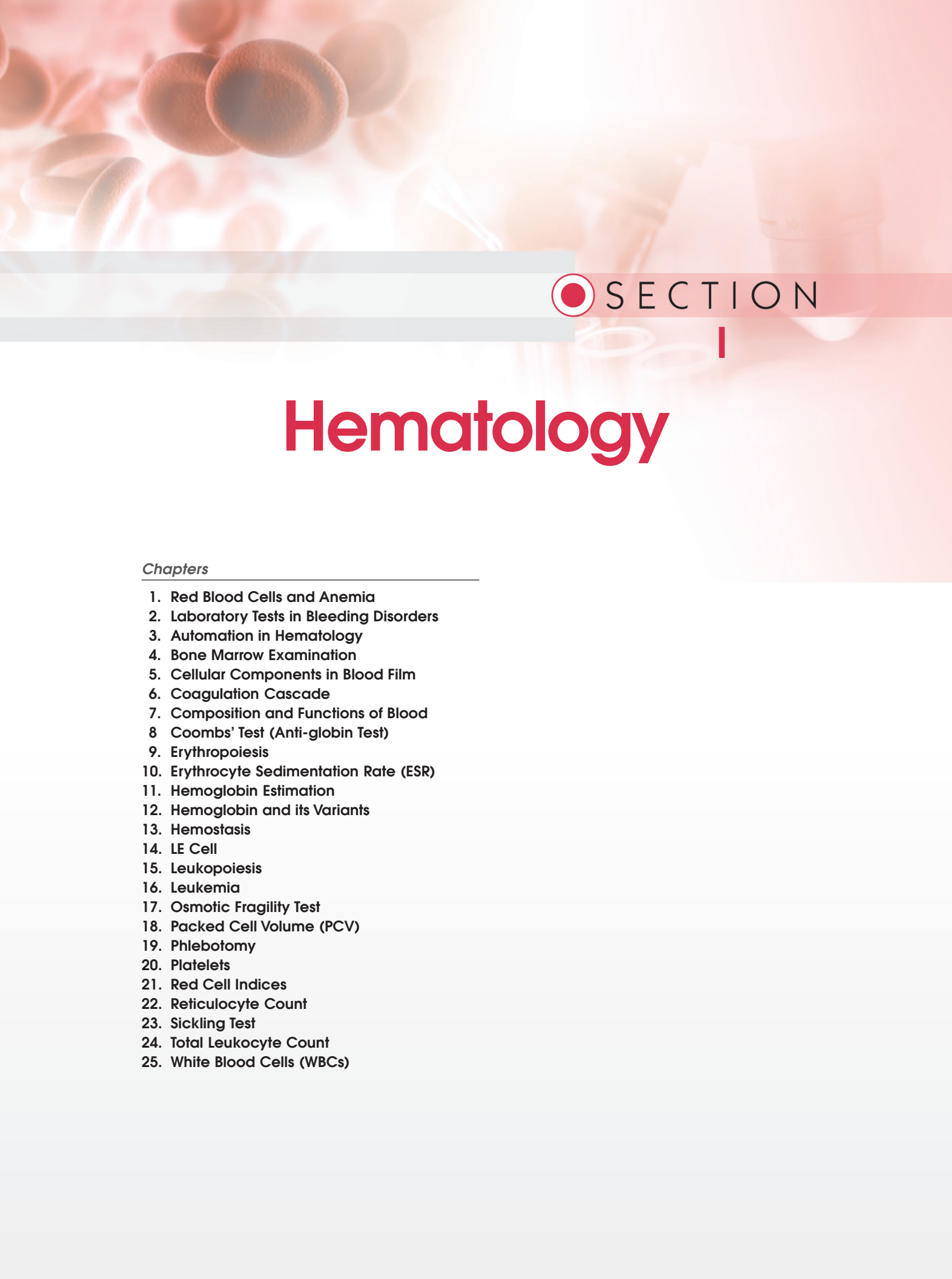




SECTION

I

Hematology

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Red Blood Cells and Anemia

RED BLOOD CELL

- RBC is 7–8 microns in size, appears round with smooth contours and stains deep pink at the periphery and paler in the center.
- Central pallor is one-third of the diameter of the RBC.

Function

Transport and delivery of oxygen to the tissues.

RBC Count

- RBC range for men is 4.7 to 6.1 million cells per microliter
- RBC range for non-pregnant women is 4.2 to 5.4 million cells per microliter.

Morphology

- Normal RBC* is normocytic (normal size) normochromic (normal staining intensity)
- RBCs with abnormal size*
 - Microcytes (small RBCs)
 - Macrocytes (large RBCs)
- RBCs with varying hemoglobin content*
 - Hypochromic (RBCs with increased area of central pallor)
 - Dimorphic (two distinct populations exist together, e.g. microcytic and macrocytic RBCs)
- RBCs with variation in shape*
 - Sick cells (RBC with both ends pointed and sickle shaped)

- Spherocytes (small RBCs show absence of central one-third of pallor)
- Schistocytes (fragmented RBCs)
- Target cells (RBCs with a central stained area and a peripheral stained rim with unstained cytoplasm in-between)

e. RBC inclusions

- Basophilic stippling (presence of numerous, basophilic granules in the RBCs)
- Howell-Jolly bodies (nuclear remnants situated peripherally on RBCs)
- Pappenheimer bodies (basophilic, small, iron containing granules in RBCs)
- Cabot's ring (figure of 8 appearing structure on RBC)

f. Immature RBCs

- Polychromatophils (young RBC with RNA material within themselves)
- Nucleated RBCs (erythroblasts)

- RBC arrangement*: Rouleaux formation: RBCs appear as stack of coins, being present on top of one another.

ANEMIA

A. Iron Deficiency Anemia

Due to reduced iron in body, there occurs reduced hemoglobin synthesis.

Peripheral Smear (Fig. 1.1)

- RBCs are microcytic (small size) and hypochromic (more than one-third of central pallor of the RBC) in chronic cases.
- Mild to moderate anisocytosis (change in cell size) and poikilocytosis (change in cell shape)
- Platelets can be normal/increased

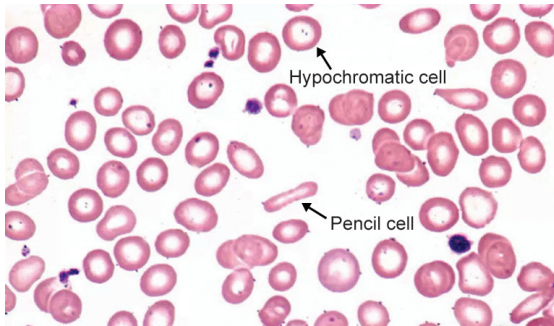


Fig. 1.1: Peripheral smear of iron deficiency anemia

B. Megaloblastic Anemia

- Occurs due to impairment of DNA synthesis and defective nuclear maturation.
- This results in nuclear to cytoplasmic asynchrony.

Peripheral Smear (Fig. 1.2)

- Shows macro-ovalocytes (large RBCs, which are oval in shape)
- Shows megaloblasts and hypersegmented neutrophils (5 lobes in 3% of neutrophils or 6 lobes in 1% of neutrophils)

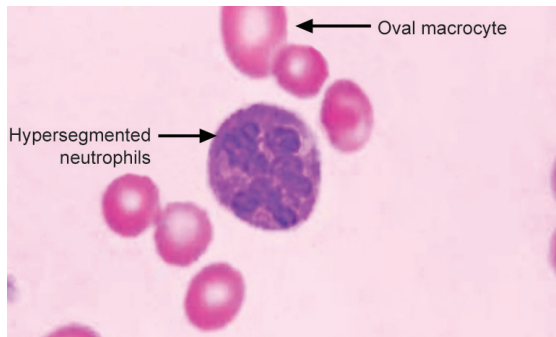


Fig. 1.2: Smear showing features of macrocytic anemia

- Can show red blood cells with multiple Howell-Jolly bodies (nuclear remnants present at the periphery of RBC)
- Reticulocyte count is low

C. Thalassemia

- Normal hemoglobin is composed of alpha and beta chains
- Deletion of these chains results in α -thalassemia and β -thalassemia.

Peripheral Smear (Fig. 1.3)

- Microcytic hypochromic RBCs
- Target cells, fragmented RBCs, nucleated RBCs, basophilic stippling and Howell-Jolly bodies (Fig. 1.4).

D. Sickle Cell Anemia

- Point mutation in β -globin gene leads to replacement of glutamate residue with a valine residue.

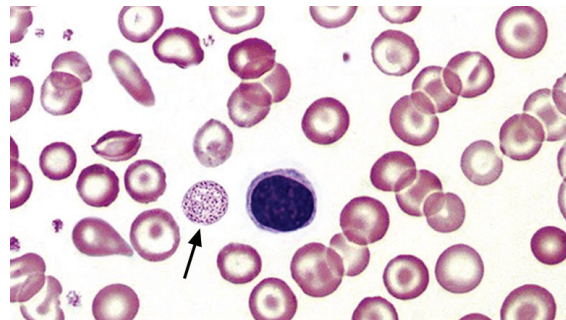


Fig. 1.3: RBCs showing microcytes, anisopoikilocytosis, target cells, basophilic stippling (arrow)

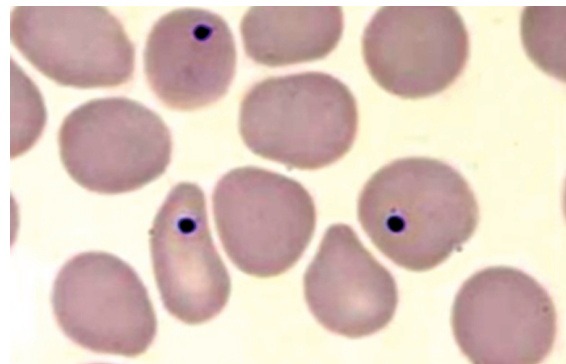


Fig. 1.4: Howell-Jolly bodies

- Results in abnormal RBC production, which when undergoes de-oxygenation becomes sickle shaped.

Peripheral Smear (Fig. 1.5)

- Sickle shaped RBCs
- Polychromatophilic RBCs
- Target cells, Howell-Jolly bodies

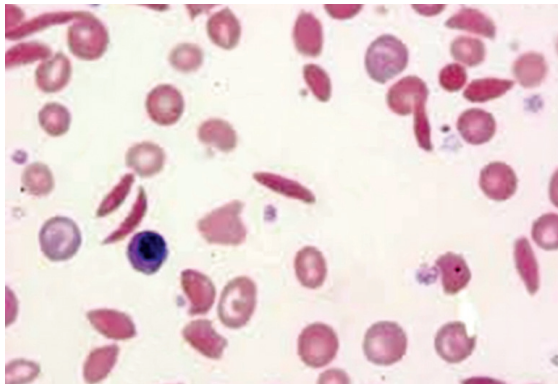


Fig. 1.5: Sickle shaped RBCs and a single nucleated (n) RBC

E. Hereditary Spherocytosis

- Occurs due to RBC membrane defect
- Defective red cell membrane components, i.e. α -spectrin, β -spectrin, ankyrin, band 4.2, or band 3 are seen.
- These mutations lead to red cells to lose membrane fragments.

Peripheral Smear (Fig. 1.6)

- Microcytic hypochromic anemia

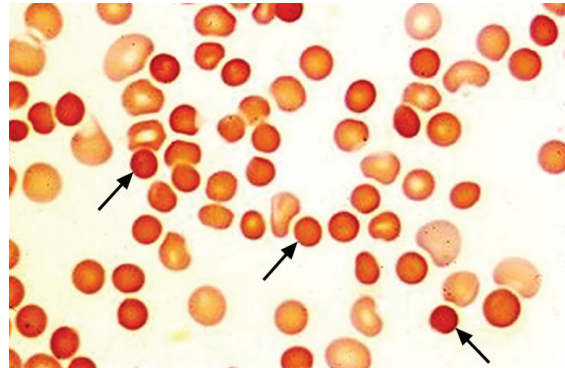


Fig. 1.6: Spherocytes in hereditary spherocytosis (arrows)

- RBCs appear small and lack central pallor (microspherocytes)
- Increased reticulocyte count

F. Glucose-6-Phosphatase Deficiency

- Occurs due to enzyme glucose-6-phosphatase dehydrogenase (G6PD) deficiency.
- Results in destruction of RBCs in the spleen and blood.

Peripheral Smear (Fig. 1.7)

- Polychromatic RBCs
- Heinz bodies (appear as dark inclusions in RBCs)
- Bite cells, which occur due to splenic removal of denatured hemoglobin.
- Splenic macrophages remove these Heinz bodies and RBCs now are called "bite cells".

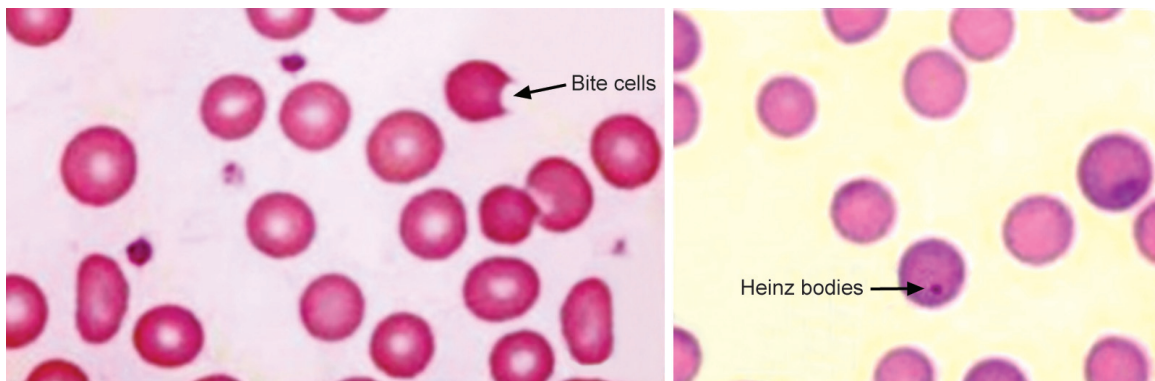


Fig. 1.7: Bite cells and Heinz bodies in G6PD deficiency smear

Laboratory Tests in Bleeding Disorders

1. BLEEDING TIME

- Assesses primary hemostasis
- Dependent on functioning of platelets and blood vessels
- A superficial skin puncture or incision is made and the time required for bleeding to stop is measured.
- Assessment is done by Ivy's method

Ivy's Method

Principle: With the help of lancet blades, three punctures are made on the dorsal surface of forearm under standard pressure, and the average time required for blood to stop oozing out from the puncture site is noted.

Equipment

- Sphygmomanometer
- Sterile disposable lancet blades (2–2.5 mm blade)
- Stop watch
- Filter paper

Methods

- A sphygmomanometer cuff is wrapped around the upper arm and inflated to 40 mm of Hg.
- Dorsal surface of forearm is cleaned with 70% ethanol and allowed to dry.

- Three punctures are made (about 5 cm apart) with lancet blade, one after another.
- A stop watch is started as soon as puncture is made.
- Blood oozing from the puncture wound is gently blotted with a filter paper at 15 seconds interval.
- Timer is stopped, when blood no longer stains the filter paper.
- Time required for bleeding to cease from all the three puncture wounds is noted.
- Average time should be taken and is reported as bleeding time.
- Puncture site is covered with a sterile adhesive strip.

Result

Reference range: 2–7 minutes.

Prolonged bleeding time is seen in

- Thrombocytopenia
- Platelet function disorders
- von Willebrand disease
- Disorders of the blood vessels

2. CLOTTING TIME

- Measures the time required for the blood to clot in a glass test tube kept at 37°C.
- Clotting time is prolonged in deficiency of clotting factor.

ASSESSED BY CAPILLARY TUBE METHOD AND LEE-WHITE METHOD

a. Capillary Tube Method

Requirements

Pricking needle, stopwatch, glass capillary tube (10 cm long), cotton swab, alcoholic swab.

Methods

- Apply alcoholic cotton swab on the fingertip. Allow it to dry naturally.
- Prick the finger, immediately stopwatch is started.
- Dip one end of the capillary tube on the drop of blood.
- Allow to fill the capillary tube with blood
- After every 30 seconds, with the use of stopwatch, break a small piece of capillary.
- Repeat breaking at regular time intervals, till fibrin thread appears at the broken end of capillary tube.
- Record time intervals between fingerprick and appearance of the fibrin thread at the broken ends of capillary tube and that is called the clotting time of blood.

b. Lee-White Method

Requirements

Water bath at 37°C, test tubes, stopwatch, syringes, fresh whole blood.

Methods

- Three-labeled glass test tubes labeled with patient's name are taken and numbered as 1, 2, and 3.
- Venepuncture is performed using a 20-gauge needle and 4 ml of blood is drawn.
- Remove the needle from the syringe, and fill each of the three tubes with 1 ml blood.
- Last 1 ml of blood is discarded
- Start the stopwatch as soon as the blood enters the syringe.
- Place the three test tubes in 37°C water bath
- At exactly 3 min, remove the first tube from water bath and tilt gently to a 45° angle to see whether the blood has clotted.

- If the blood is not clotted, return it to the water bath and examine it at 30 seconds intervals.
- After the blood in the first tube has clotted, examine the second tube immediately.
- Then examine the 3rd tube.
- Record the time it took the blood in the 3rd test tube to clot.

Result

Normal clotting time: 2 to 9 minutes.

3. PROTHROMBIN TIME (PT)

Assesses the coagulation factors in extrinsic pathway (factor VII) and common pathway (factor X, V, prothrombin, fibrinogen).

Principle (Fig. 2.1)

Tissue thromboplastin and calcium are added to plasma and clotting time is determined.

Equipment

- Water bath at 37°C

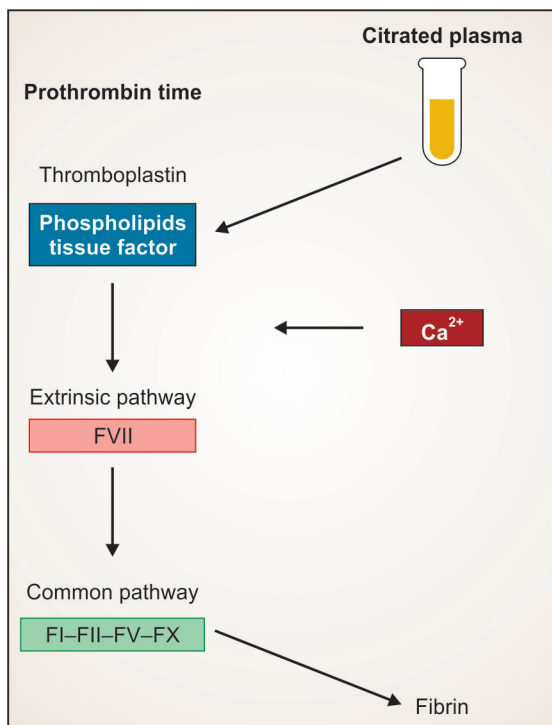


Fig. 2.1: Principle of prothrombin time

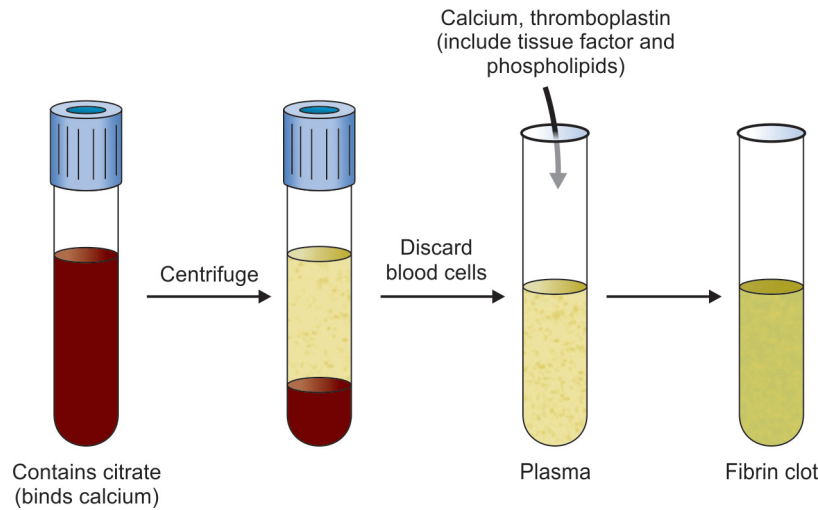


Fig. 2.2: Prothrombin time procedure

- Test tubes
- Stopwatch

Reagents

- Thromboplastin reagent contains tissue factor
- Calcium chloride

Specimen

Platelet poor citrated plasma.

Methods (Fig. 2.2)

- Anticoagulated blood is centrifuged at 3000–4000 revolutions per minute for 15–30 minutes.
- Plasma is separated and 0.1 ml is taken in glass test tube and is kept in water bath at 37°C.
- Add 0.1 ml of thromboplastin reagent and mix.
- After 1 minute, add 0.1 ml of calcium chloride solution.
- Start the stopwatch and record the time required for clot formation.

Normal Range

11–16 seconds

Prolongation of PT is seen in

- Patients taking oral anticoagulants

- Liver disease
- Vitamin K deficiency

4. ACTIVATED PARTIAL THROMBOPLASTIN TIME (APTT)

Assesses the coagulation factors in intrinsic pathway (factors XII, XI, IX, VIII) and common pathway (factors X, V, prothrombin and fibrinogen).

Principles (Fig. 2.3)

- Plasma is incubated with an activator, thus initiating the intrinsic pathway of coagulation
- Phospholipid and calcium are now added and time for clot formation is noted.

Reagents

- *Kaolin*: Contact activator
- Phospholipid
- Calcium chloride

Specimen

Platelet poor citrated plasma.

Methods (Fig. 2.4)

- Mix equal volumes of phospholipid reagent and calcium chloride solution in a test tube and keep in a water bath at 37°C.

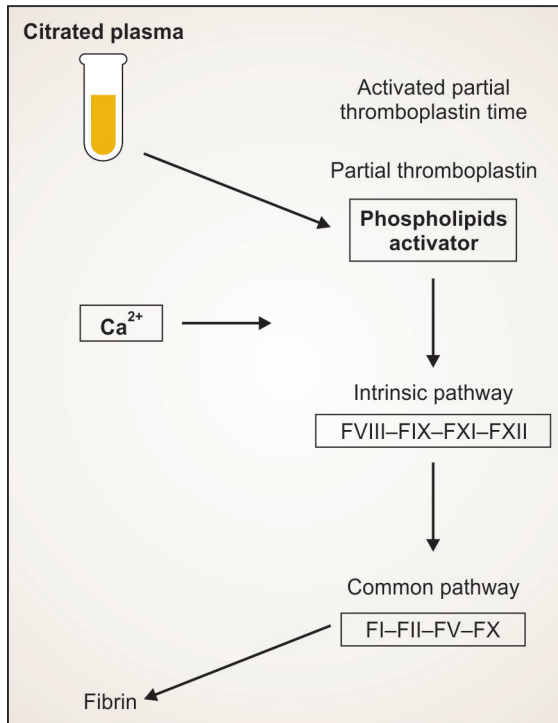


Fig. 2.3: Principle of APTT

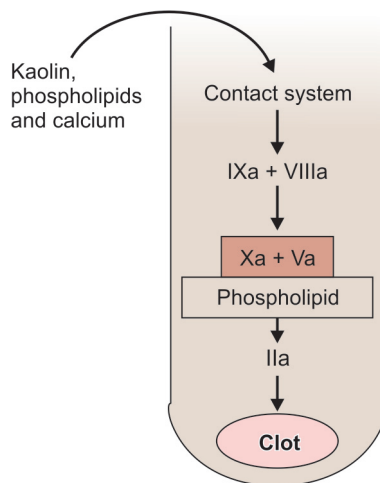


Fig. 2.4: APTT procedure

- Take 0.1 ml of platelet poor citrated plasma in another test tube and add 0.1 ml of kaolin solution.

- Incubate at 37°C in water bath for 10 minutes
- After 10 minutes, add 0.2 ml of phospholipid-calcium chloride mixture, and with the help of stopwatch, note the clotting time.

Normal Range

30–40 seconds

Prolonged APTT is seen in

- Hemophilia A or B
- Heparin therapy
- Liver disease
- Deficiencies of coagulation factors in intrinsic and common pathways.

5. THROMBIN TIME

Assesses the final step of coagulation, i.e. conversion of fibrinogen to fibrin by thrombin.

Principle

Thrombin is added to patients plasma and time required for clot formation is noted.

Reagent

Thrombin solution.

Specimen

Citrated platelet poor plasma.

Methods

- Take 0.1 ml of buffered saline in a test tube and add 0.1 ml of plasma.
- Clotting time is noted after addition of 0.1 ml of thrombin solution.

Normal Range

3 seconds.

Prolonged thrombin time is seen in

- Disorders of fibrinogen: Afibrinogenemia, hypofibrinogenemia, dysfibrinogenemia.
- Heparin therapy (heparin inhibits action of thrombin).

Automation in Hematology

What is Automation?

Automation is the process in which the computerized system has taken over the work performed by humans in laboratories.

INTRODUCTION

The hematological tests, which were performed by manual methods, had been replaced by machines.

At the peripheral centers, still manual methods are being used. Hematology analyzers, which generate the results at a fast rate and without errors, have widely replaced manual tests like WBC count, RBC count, platelet count, PCV, ESR, etc.

How is Automation Useful?

- Test reports can be obtained at a fast rate
- Large number of samples can run simultaneously
- Multiple tests can be interpreted with a single sample
- High accuracy of these test results as compared to tests performed manually
- Reduction in manpower
- Reducing the operator exposure to potential hazardous biological material.

Disadvantages of Automation

- Flagged reports (which require slide examination for confirmation)

- Expensive with high running costs
- Interfering factors for various test results.

Three stages of laboratory testing are

- a. Pre-analytical
- b. Analytical
- c. Post-analytical

a. Pre-analytical Stage

- Concerned with sample or specimen processing
- Printed bar code labels are used for sample labeling
- Bar code labeling has reduced processing times and pre-analytical errors.
- Proper attention has been attributed for the steps of centrifugation, decapping, aliquoting, recapping, storage and retrieval.

b. Analytical Stage

Tasks and remedies included in this phase include:

1. *Sample introduction into the automation machine*

- Sample being taken up by the sample probe from the vacutainer.
- Sample probes have a clot detection property, as clot can result in malfunction of the automated analyzer.
- Carry over contamination with one sample from another can be avoided by usage of wash solution in-between each pipetting.

2. Reagents

- Automated systems are classified as open reagent systems and close reagent systems.
- Open reagent system is one, in which reagents other than the instrument manufacturer's reagents can be used and these systems are more flexible.
- Closed reagent system is one, in which only instrument manufacturer's reagents can be used and is a more expensive system.
- For an individual test, a fixed proportion of sample volume and reagent volume should be used.

3. Incubation

- A proper temperature should be maintained in a system for proper test results.
- This can be achieved with the help of heat pumps or thermal rings inside the machine.

4. Detection

- Absorption spectroscopy is used in automated analyzers.
- Principle used in automation system for detection is electrochemiluminescence

c. Post-analytical Stage

- After a test sample is run, the electrical signals from the detector are interpreted by the computers as a digital signal.
- Multiple signals are interpreted and the results are displayed both as data and figures.

Parameters Measured by Hematology Analyzers

- RBC count
- Hemoglobin
- Mean cell volume
- Mean cell hemoglobin
- Mean cell hemoglobin concentration
- WBC count
- WBC differential
- Platelet count
- Red cell distribution width
- Reticulocyte count
- Mean platelet volume
- Platelet distribution width

Bone Marrow Examination

Bone marrow is the site of hematopoiesis in postnatal life.

During **infancy and early childhood**, hematopoiesis is seen in all bones of the body.

Ribs, sternum, iliac bones, vertebrae, proximal end of long bones are the major sites of hematopoiesis in **late childhood**.

Sites of Bone Marrow Aspiration

Iliac crest, sternum, tibia, spinous processes of lumbar vertebrae.

In **bone marrow aspiration**: Bone marrow fluid is aspirated and smears are prepared on glass slides.

In **bone marrow biopsy (trephine biopsy)**: Tissue of bone marrow is removed and processed in histopathology laboratory.

Indications for Bone Marrow Aspiration

- Unexplained cytopenias
- Suspected acute leukemia for classification and categorization.
- Suspected chronic myeloproliferative disorders.
- Suspected myelodysplastic syndromes
- Metastatic tumors to the marrow
- Investigation of pyrexia of unknown origin
- Suspected storage disorder like Gaucher's disease or Niemann-Pick disease.
- Suspected infections like kala-azar, miliary tuberculosis, or histoplasmosis.

Indications for Bone Marrow Biopsy

- Dry tap as seen in myelofibrosis or leukemia
- Suspected aplastic anemia
- Suspected myelofibrosis
- Suspected hairy cell leukemia
- Staging of lymphoma

Processing of Marrow Specimens

1. Bone marrow aspiration

- Drop of a sample is placed on a slide
- Smears are prepared by a spreader
- Marrow particles are carried behind the spreader and cellular trails are produced while spreading.
- Smears are dried and stained with Romanowsky stains.

2. Bone marrow trephine biopsy

- Biopsy specimen is fixed with 10% formalin
- Biopsy is processed to obtain paraffin wax blocks.
- 4 microns thick sections, 5 in number are cut in a stepwise fashion.
- These sections are stained with hematoxylin and eosin.
- Prussian blue, Giemsa stain will be helpful in diagnosis.

Information Obtained from Bone Marrow Aspiration

1. Morphology
2. Cytochemistry
3. Iron stain
4. Immunophenotyping
5. Culture

Studies which can be done on bone marrow aspirate smears

- Romanowsky stain
- Iron stain
- Cytochemistry
- Molecular genetics
- Immunophenotyping
- Culture

Information received from bone marrow biopsy

1. Cellularity (normocellular, hypercellular or hypocellular)
2. Architecture (well preserved or poorly preserved)
3. Fibrosis (present or absent)
4. Focal lesions (present or absent)
5. Bone structure (preserved or lost)

Studies which can be done on bone marrow biopsy

- Hematoxylin and eosin stain
- Reticulin stain
- Immunohistochemistry

Cellular Components in Blood Film

Peripheral blood smear is examined for

- Red blood cells morphology
- White blood cells*: Differential count
- Platelet count
- Parasites*: Malaria, filaria

Peripheral blood smear comprises three parts: Head, body and tail.

1. Red Blood Cells (RBCs)

- Examined at the tail end of smear
- Normal red cells are 7–8 microns in size with an area of central pallor.
- Size of normal red cell corresponds to the size of the nucleus of a small lymphocyte.

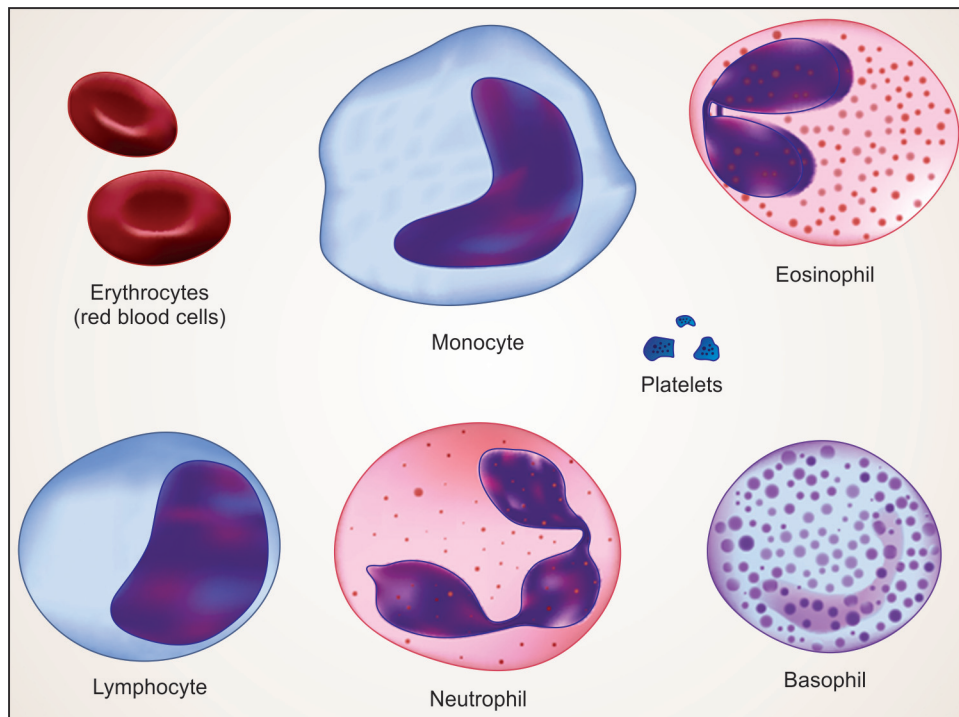


Fig. 5.1: Types of blood cells

- In normal blood smear, RBCs appear normocytic normochromic.
- RBCs smaller than normal are called microcytes.
- RBCs larger than normal are called macrocytes.
- RBCs which are large and oval in shape are called macro-ovalocytes.

2. White Blood Cells (WBCs)

Differential leukocyte count (DLC) comprises neutrophils, lymphocytes, eosinophils, basophils and monocytes (Fig. 5.1).

a. Polymorphonuclear neutrophils

- Neutrophil measures 14–15 microns in size
- Cytoplasm contains granules
- Nucleus has 2–5 lobes
- Normal count is 40–75%

b. Eosinophils

- Measures 15–16 microns
- Bilobed nucleus and cytoplasm have large orange-red granules
- Normal count is 1–6%

c. Basophils

- Measures 9–12 microns
- Cytoplasm contains large, coarse, purple granules obscuring the nucleus.
- Normal count is 0–1%

d. Monocytes

- Largest leukocytes
- Measures 15–20 microns
- Cells have abundant cytoplasm and irregular shape oval or clefted nucleus.
- Normal count is 2–10%

e. Lymphocytes

- Two types of lymphocytes—small and large
- Small lymphocytes are 7–8 microns in size, with blue cytoplasm.
- Large lymphocytes are 10–15 microns in size, with pale blue cytoplasm.
- Normal count is 20–40%.

3. Platelets

Small, 1–3 microns in diameter, purple structures.

Coagulation Cascade

A. In laboratory (Fig. 6.1)

Intrinsic pathway: Clotting is initiated by phospholipids, calcium, and negatively charged substance such as glass beads.

Extrinsic pathway: Clotting is initiated with source of tissue factor.

B. In blood vessel: Tissue factor is the major initiator of coagulation.

Assessment of coagulation pathway is done by

- Prothrombin time (PT) assay:* Assesses the function of proteins in the extrinsic pathway (factors VII, X, V, II, and fibrinogen)
- Partial thromboplastin time (PTT) assay:* Assesses the function of the proteins in the intrinsic pathway (factors XII, XI, IX, VIII, X, V, II, and fibrinogen)

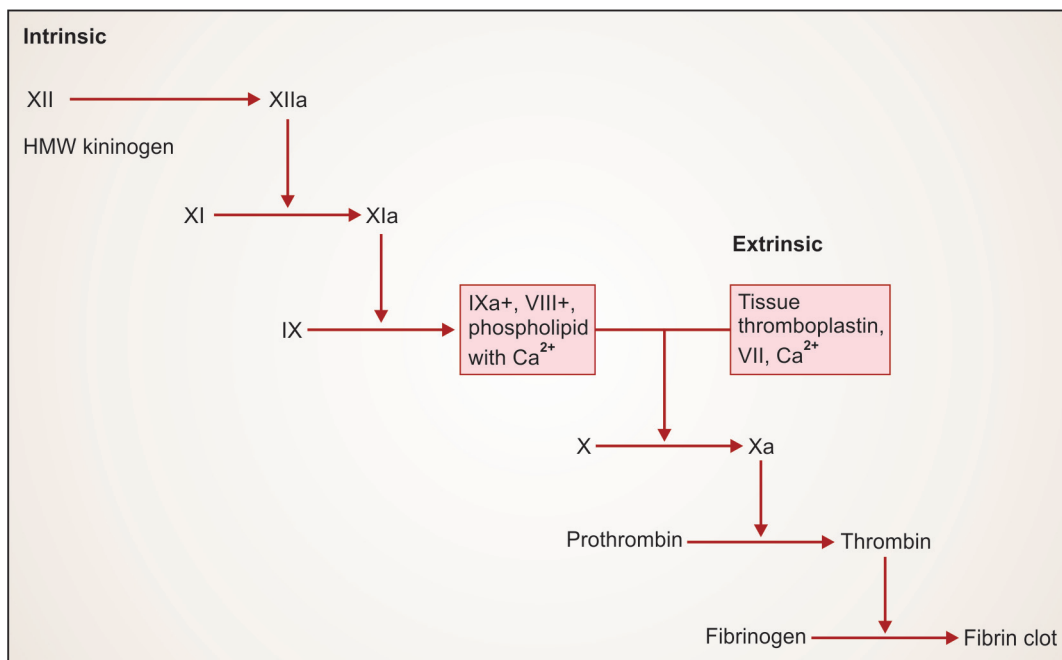


Fig. 6.1: Coagulation cascade

COAGULATION DISORDERS

A. Inherited Disorders

Hemophilia A (Factor VIII Deficiency)

- Most common hereditary disease associated with life-threatening bleeding, caused by mutations in factor VIII.
- Patient presents with spontaneous hemorrhages into joints (hemarthrosis).
- Patients have prolonged PTT and a normal PT.

Hemophilia B (Christmas Disease, Factor IX Deficiency)

- Factors VIII and IX function together to activate factor X.
- PTT is prolonged and the PT is normal.

B. Acquired Disorders

Vitamin K deficiency and liver disorders result in the impaired synthesis of factors II, VII, IX, X and protein C.

ANTICOAGULANTS

- Laboratory instruments, blood transfusion bags, medical and surgical pieces of equipment will become non-functional, if the anticoagulant is not used as the blood will clot inside them.

- These anticoagulants inhibit calcium ions and thus inhibit the coagulant cascade.

Examples

- EDTA (ethylenediaminetetra-acetic acid) binds and chelates calcium
- Sodium citrate
- Oxalate

Citrate containing anticoagulants: Acid citrate dextrose, citrate phosphate dextrose, sodium citrate, citrate phosphate dextrose with adenine (CPD-A), Alsever's solution.

Anticoagulants used according to the vacutainer used

- *Blue vacutainer (used for coagulation studies):* 3.2% trisodium citrate (2.7 ml of blood + 0.3 ml of anticoagulant = 9:1)
- *Lavender (for routine hematological investigations):* Dipotassium EDTA
- *Grey (for blood glucose):* Dipotassium EDTA contains sodium fluoride (antimetabolite)
- *Black (for ESR):* 3.8% trisodium citrate (1.6 ml of blood and 0.4 ml of citrate = 4:1)
- *Red (for serum studies):* Without anticoagulant (4 ml of blood is collected)
- *Green for enzyme studies, osmotic fragility test, arterial blood gas analysis* contains lithium heparin.

Composition and Functions of Blood

1. COMPOSITION OF BLOOD

Blood is made up of plasma, RBCs, WBCs and platelets.

a. *Plasma*

- Straw-colored fluid
- 90% is water and also contains electrolytes such as sodium, potassium and proteins.

b. *RBCs*

- Main function of RBCs is to carry oxygen.
- RBC has a lifespan of 120 days, before it is broken down in spleen.

c. *WBCs*: Comprises neutrophils, lymphocytes, monocytes, eosinophils and platelets.

d. *Platelets*: Disc-shaped fragments involved in clotting of blood.

2. FUNCTIONS OF BLOOD

a. *Transportation*

- Blood carries gases (CO_2 , O_2) between the lungs and rest of the body.

- Nutrients from the digestive tract from site of absorption to the body.
- Waste products to be detoxified by the liver or kidneys.
- Hormones from the glands in which they are produced to their target cells.
- Provides heat and warmth to the skin to regulate the body temperature.

b. *Maintaining hemostasis*

c. *Protection*: Blood plays a role in inflammation.

- WBCs destroy the cancer cells
- Antibodies destroy the pathogens
- Platelets initiate the clotting and help in minimizing the blood loss.

d. *Regulation*: Blood helps regulate

- pH by interaction between acids and bases.
- Water balance by transferring water to and from the tissues.

Coombs' Test (Anti-globin Test)

Two types

- Direct antiglobulin test
- Indirect antiglobulin test

a. Direct Antiglobulin (Coombs') Test (DAT) (Fig. 8.1)

- Detects antibodies or complement or both attached to the red cells.
- Principle: Red cells of the patient are washed with normal saline (to remove unbound antibodies) and anti-human globulin (AHG) reagent is added.
- Agglutination of red cells indicates positive test.

Causes of positive test: Autoimmune hemolytic anemia, hemolytic diseases of newborn,

hemolytic transfusion reaction, drug-induced hemolysis.

b. Indirect Antiglobulin (Coombs') Test (IAT) (Fig. 8.2)

- Detects the presence of antibodies in the serum, directed against the red cell antigens.
- Patient's serum is incubated with red cells (donor or screening) to allow binding of antibodies in serum to red cell antigens.
- After washing of red cells in saline (to remove unbound antibodies), anti-human globulin reagent is added.
- Agglutination of the red cells denotes positive test.

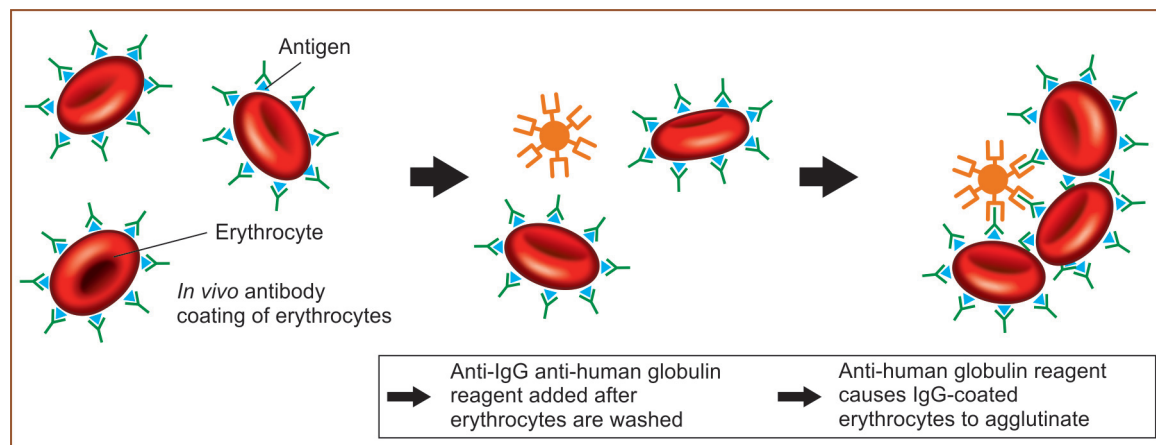


Fig. 8.1: Direct Coombs' test

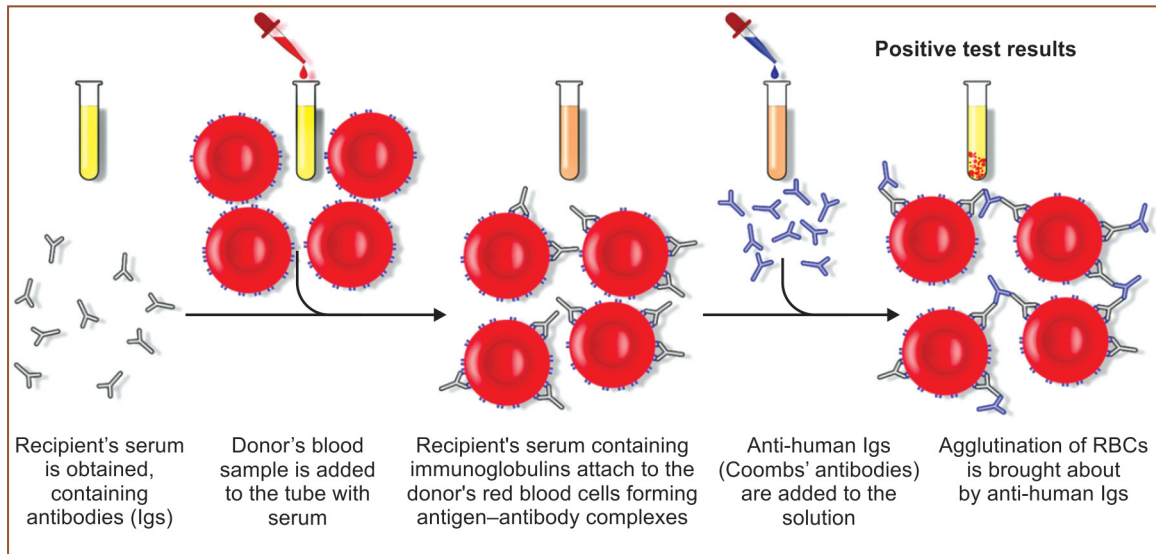


Fig. 8.2: Indirect Coombs' test

Uses of indirect agglutination test

- Cross-matching before transfusion
- Antibody screening and identification
- For detecting anti-Rh antibodies in the serum.

Erythropoiesis

Stages of Erythropoiesis (Fig. 9.1)

1. **Pro-erythroblast:** Large cell, open chromatin, with blue cytoplasm.
2. **Basophilic (early) erythroblast:** Fine nuclear clumped chromatin.
3. **Intermediate erythroblast (polychromatophilic):** Smaller cell with coarse clumped chromatin.
4. **Late erythroblast (orthochromatic):** Small, dense, pyknotic and eccentric nucleus
5. **Reticulocyte (polychromatic RBC):** No nucleus and blue-gray color cytoplasm.
6. **Erythrocytes:** No nucleus and red cytoplasm.

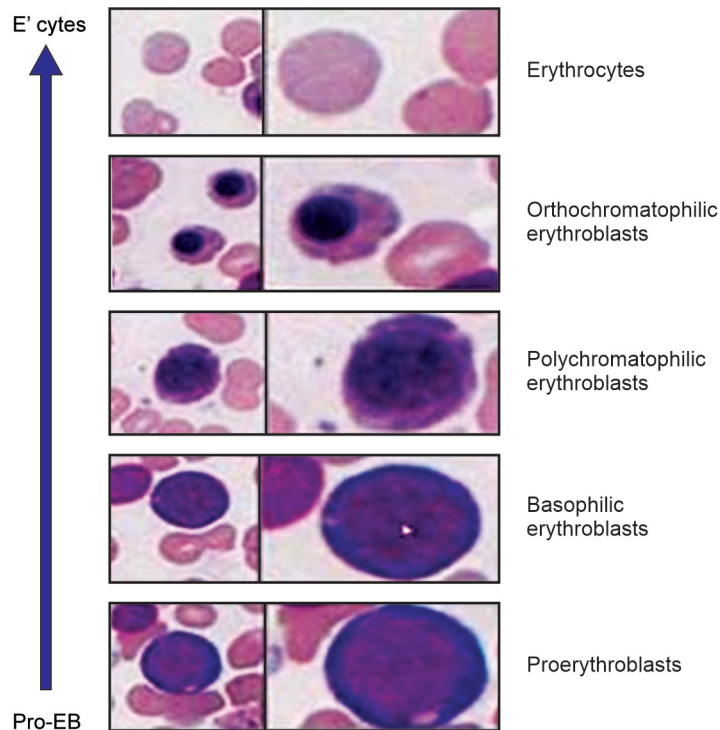


Fig. 9.1: Stages of erythropoiesis

Erythrocyte Sedimentation Rate (ESR)

- ESR measures the rate of sedimentation (settling) of erythrocytes in the anti-coagulated whole blood.
- Anticoagulated blood sample is allowed to stand in a glass tube for 1 hour.

Stages of ESR

Three stages:

- **Stage 1: Formation of Rouleaux (10 minutes):** Red cells stack together like a pack of coins
- **Stage 2: Sinking of Rouleaux (40 minutes):** Rapid and constant sedimentation.
- **Stage 3: Packing of Rouleaux (10 minutes):** Slow sedimentation.

Why is ESR Measured?

- Measurement is indicated in cases of infections, inflammatory disorders or cancers.
- To monitor the disease activity in tuberculosis, arthritis.

Methods of Estimation of ESR

- Westergren method
- Wintrobe method
- Zeta sedimentation ratio
- Micro-ESR.

Westergren Method

Equipment and reagents:

1. **Westergren's ESR tube** (Fig. 10.1)

- Straight glass pipette measuring 300 mm in length
- Calibrated in mm from 0 to 200 (top to bottom)
- Internal diameter is around 2.5 mm
- Tube should be dry and clean

2. **Westergren stand:** Holds the tube in a motionless, vertical position.

3. **Anticoagulant diluted solution:** Tri-sodium citrate dihydrate is the anticoagulant of choice.

Specimen Collection

Venous blood is collected in trisodium citrate solution in the ratio of 4:1 (blood:citrate).

Methods

- Mix anticoagulated blood thoroughly
- Westergren tube is filled with the blood sample up to the zero mark.
- There should be no air bubbles while filling the tube.
- Tube is placed in a vertical position in ESR stand and is left undisturbed for 1 hour.
- After 1 hour, read the height of the column of plasma above the red cell column in mm.

Result

Express the result as erythrocyte sedimentation rate = mm in 1 hour.

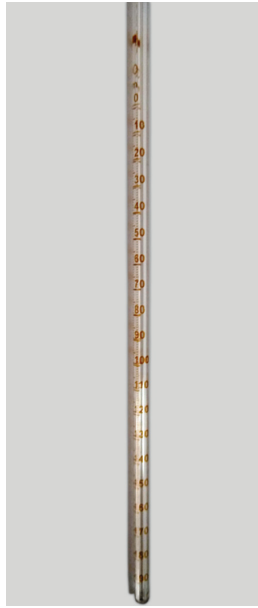


Fig. 10.1: Westergren's tube

Following points should be taken care of

- Test should be performed at room temperature
- Use the correct proportion of blood and anticoagulant
- ESR tube should be in a vertical position.

Normal Values

- Males <50 years: 0–15 mm in 1 hour
- Females <50 years: 0–20 mm in 1 hour
- Children: 0–10 mm in 1 hour.

Hemoglobin Estimation

INDICATIONS FOR HEMOGLOBIN ESTIMATION

- To determine the presence and severity of anemia: Anemia refers to low hemoglobin levels.
- To diagnose polycythemia, i.e. increased hemoglobin levels.
- To monitor the response of treatment in patients with anemia.
- To estimate red cell indices.

METHODS OF ESTIMATION OF HEMOGLOBIN

- Calorimetric method:
 - Sahli's acid hematin method
 - Cyanmethemoglobin method
- Gasometric method
- Chemical methods
- Specific gravity method.

a. Sahli's Acid Hematin Method

Principles

- Blood is mixed with acid solution, which converts hemoglobin into brown-colored acid hematin.
- This solution is diluted with water till the brown color matches that of brown glass standard.
- Hemoglobin value is read directly from the scale.

Equipment

- Sahli's hemoglobinometer consists of Sahli's hemoglobin tube (marked in gm%) and a comparator with a brown glass standard.
- Sahli's pipette or hemoglobin pipette (marked as 20 microliter or 0.02 ml).
- Stirrer
- Pipette

Reagents

- N/10 hydrochloric acid
- Distilled water

Specimen

EDTA blood

Methods

- Put N/10 HCl into Sahli's graduated hemoglobin tube up to the mark of 2 gm
- Take the blood sample in Sahli's pipette up to 20 microliters mark.
- Add blood sample to the acid solution
- Mix with a glass stirrer
- Allow it to stand for 10 minutes
- Add distilled water drop by drop till the color of the solution matches that of brown glass standard.
- Take the reading of the lower meniscus from the tube in grams.

Disadvantages of Sahli's Method

- For maximum development of color of acid hematin, it takes an hour, however, 95% of the color is attained in 10 minutes and the reading in our test is taken at the end of ten minutes.
- HbF seen in infants will not be converted to acid hematin on treatment with HCl, hence this method is not used for detecting hemoglobin of infants.
- Matching color with brown glass can give false interpretation of the results and hence false values.

b. Cyanmethemoglobin Method

Method of choice for estimation of hemoglobin as all forms of hemoglobin are converted to cyanmethemoglobin.

Reagents**1. Drabkin's solution**

- Potassium ferricyanide—200 mg
- Potassium cyanide—50 mg
- Potassium dihydrogen phosphate—140 mg
- Non-ionic detergent—1 ml
- Distilled water to make 1000 ml

2. Cyanmethemoglobin standard solution with known hemoglobin value.**Principles**

- Blood is mixed with Drabkin's solution
- Erythrocytes are lysed producing hemoglobin.
- Potassium ferricyanide converts hemoglobin to methemoglobin, which combines with potassium cyanide to form cyanmethemoglobin.

- Absorbance of the solution is measured in a spectrophotometer at 540 nm wavelength.
- To know the amount of hemoglobin in unknown sample, its absorbance is compared with standard cyanmethemoglobin solution.

Equipment

1. Photoelectric calorimeter or spectrophotometer
2. Sahli's pipette marked at 20 cu mm
3. Pipette 5 ml

Specimen

EDTA blood

Methods

- In a test tube, take 5 ml of Drabkin's solution and add 20 cu mm of blood (dilution factor of 1:251).
- Mix the solution by inverting several times and allow it to stand for 5 minutes.
- Transfer the test sample to a cuvette and read the absorbance in a spectrophotometer at 540 nm.
- Also note the absorption of standard solution, which is read against a reagent blank (i.e. Drabkin's solution)

Hemoglobin in gm/dl =

$$\frac{\text{Absorbance of test sample}}{\text{Absorbance of the standard}} \times \frac{\text{Concentration of standard}}{100} \times \text{Dilution factor}$$

Hemoglobin and its Variants

- Hemoglobin is made up of heme and globin
- Globin protein is composed of different types of chains, named alpha, beta, delta and gamma.

Normal hemoglobin types include

- a. *Hemoglobin A*: Makes up 95–98% of hemoglobin in adults, contains two alpha (α) and two beta (β) protein chains.
- b. *Hemoglobin A₂*: Makes up 2–3% of hemoglobin found in adults, contains two alpha (α) and two delta (δ) protein chains.
- c. *Hemoglobin F*: Makes up 1–2% of hemoglobin, found in adults, contains two alpha (α) and two gamma (γ) protein chains.

Hemoglobin Variants

- Globin gene mutation, results in structurally altered hemoglobin like HbS (sickle cell anemia).
- Globin gene mutation, results in altered hemoglobin production as seen in thalassemia.
- HbE disease and HbE trait
- HbC disease and HbC trait
- *Hemoglobin F (HbF)*: Elevated levels are seen in beta-thalassemia, sickle cell anemia, or in hereditary persistence of fetal hemoglobin.
- *Hemoglobin H (HbH)*: Seen in patients of alpha-thalassemia, and are composed of

four beta (β) globin chains, produced due to shortage of alpha (α) chains.

- *Hemoglobin Bart*: Seen in patients of alpha-thalassemia, and are composed of four gamma (γ) chains, produced due to shortage of alpha (α) chains.
- *Hemoglobin SC disease*: Inheritance of one beta S gene and one beta C gene.
- *Sickle cell—hemoglobin D disease*: Inheritance of one hemoglobin S gene and one hemoglobin D—Los Angeles (or D-Punjab) gene.
- *Hemoglobin E—beta-thalassemia disease*: Individuals are heterozygous for hemoglobin E gene and beta-thalassemia gene.

Thalassemia

Clinical Course

- Due to deficient hemoglobin chain synthesis, there occurs diminished survival of RBCs.
- Also these, unpaired alpha chains in alpha-thalassemia and beta chains in beta-thalassemia form RBC inclusions.
- Inclusions lead to membrane damage, which result in RBC destruction.
- Due to anemia, patient requires frequent blood transfusions.
- Children, who are not treated with blood transfusion, die at an early age.
- Frequent blood transfusion results in iron overload in patients blood and in their organs.

- Cardiac disease is an important cause of death, due to progressive iron overload.
- Hematopoietic stem cell transplantation can offer cure.

Diagnosis

Peripheral Smear

- Microcytic hypochromic anemia
- Marked variation in size (anisocytosis) and shape (poikilocytosis) of RBCs
- Target cells (hemoglobin collects in the center of the cell), basophilic stippling, and fragmented red cells are seen.

Levels of normal hemoglobin components and its variants in different disorders

- HbA₂ is elevated (4–8%) in beta-thalassemia trait
- HbF will be elevated in thalassemia
- HbA will be reduced in thalassemia
- HbS will be seen in sickle cell anemia.

HEMOGLOBIN ELECTROPHORESIS

Electrophoresis is the movement of hemoglobin proteins in an electric field at a fixed pH.

Principle

An electric field is applied through a supporting medium and the solutes migrate in that field, according to their net charge.

Example

For thalassemia, alkaline electrophoresis is performed.

Principles of Hemoglobin Electrophoresis (Fig. 12.1)

- It is performed to find out abnormal forms of hemoglobin (hemoglobinopathy).
- Different types of hemoglobin separated include HbA, HbA₂, HbF, HbS, HbC.
- Tissue extracts are introduced into the sample wells in gel.
- An electrical field is applied, and proteins with net negative charge will migrate towards anode end of the field.

Cellulose Acetate Method at Alkaline pH

Principles

- In an alkaline pH (8.2–8.6), hemoglobin is negatively charged molecule and migrate towards the anode.
- Various hemoglobin molecules move at different rates depending on their net negative charge, which in turn is controlled by the composition (amino acids) of the Hb molecule (globin chain).
- One end of cellulose acetate strip is immersed in the buffer (pH 8.2–8.6) on the cathode side and the other end is placed in the buffer on the anode side.
- An electric current of specific voltage is allowed to run.
- During electrophoresis, hemoglobin molecules migrate toward the anode because of their negative charge.
- Cellulose acetate membrane is then stained in order to color the proteins (hemoglobin).
- By noting the distance, each hemoglobin has migrated, and comparing this distance with

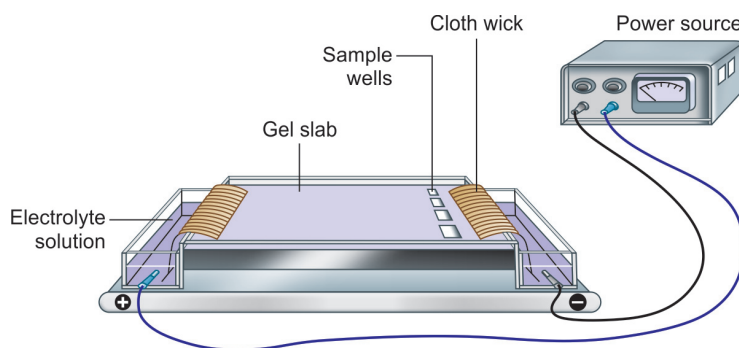


Fig. 12.1: Principle of electrophoresis

the migration distance of known controls, the types of hemoglobin may be identified.

Migration of Hemoglobin in Electrophoresis (Fig. 12.2)

- Of the normal hemoglobin in an adult, HbA is the fastest, followed by HbF

Cathode(-)	Anode(+)		
Normal	HbA ₂	HbF	HbA
Abnormal	HbC	HbS	Bart's HbH

Fig. 12.2: Pattern of distribution of abnormal hemoglobin on electrophoresis

- HbA₂ moves only slightly from the point of origin near the anode.
- Abnormal hemoglobin shows the following migration patterns: HbC migrates with HbA₂ near the anode.
- HbS lies between hemoglobin A₂ and hemoglobin F.
- HbH and Bart are unstable and very fast moving, placing them past HbA.

Note: All hemoglobin specimens that show an abnormal electrophoresis pattern in alkaline media (cellulose acetate agar) should undergo electrophoresis on an acid citrate agar.

Hemostasis

Definition: Mechanism by which blood clots at the site of vascular injury.

Sequence of Events (Fig. 13.1)

a. **Arteriolar vasoconstriction:** Arteriole, where there occurs an injury, will undergo constriction.

b. **Primary hemostasis**

- Brought about by platelets
- Platelets adhere to the vessel wall and get activated.
- Activated platelets will release mediators and bring about platelet aggregation and formation of primary hemostatic plug.

c. **Secondary hemostasis**

- Tissue factor released at the endothelial cell injury site will result in activation of coagulation pathway.
- This activation of coagulation cascade results in formation of secondary hemostatic plug, due to deposition of fibrin.

d. **Clot stabilization:** Tissue plasminogen activator limits the clot size at the site of injury.

PLATELET DISORDERS

- Following vascular injury, platelets adhere to the vWF exposed due to endothelial cell damage.

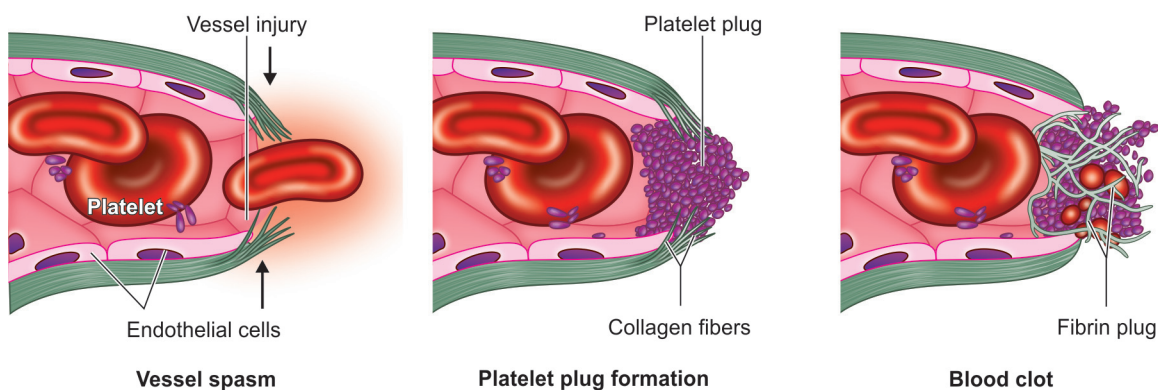
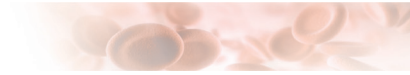


Fig. 13.1: Formation of hemostatic plug



- Platelets adhere to vWF through platelet surface receptor glycoprotein (Gp)Ib-IXa.

von Willebrand Disease

- Occurs due to deficiency of vWF
- Most common inherited bleeding disorder of humans.
- Spontaneous bleeding from mucous membranes (e.g. epistaxis), excessive bleeding from wounds, or menorrhagia.

Bernard-Soulier Syndrome

- Occurs due to deficiency of GpIb-IXa, a receptor for vWF.
- GpIb-IXa is essential for normal platelet adhesion to the subendothelial extracellular matrix.

- Bleeding due to defective platelet adhesion to subendothelial matrix.

Activated platelets produce thromboxane A_2 , which brings about platelet aggregation. Platelets have GpIIb-IIIa receptors on their surface, which increases their affinity for fibrinogen.

Glycoprotein IIb-IIIa leads to binding of fibrinogen and forms bridges among platelets, bringing about platelet aggregation.

Glanzmann Thrombasthenia

- Bleeding disorder due to inherited deficiency of GpIIb-IIIa, due to defective platelet aggregation.
- Glycoprotein IIb-IIIa (acts as a bridge between platelets by binding fibrinogen).

LE Cell

- LE cell is usually a neutrophil/polymorph that has ingested the altered nucleus of another polymorph.
- LE cell appears as basophilic, homogeneous mass that stains purplish brown.
- Lobes of the ingesting polymorph appear wrapped around the ingested material.
- Polymorphs can collect around an altered nuclear material and form a "rosette".

LE cells can be seen in

- Systemic lupus erythematosus
- Rheumatoid arthritis
- Discoid lupus erythematosus.

Methods of Demonstration of LE Cell

- Clotted blood
- Defibrinated blood
- Citrated or heparinized blood
- Rotary method.

The Rotary Method

1. 1 ml of patient's blood collected in heparin is transferred into a glass tube.
2. Four glass beads are added and the tube is sealed.
3. Preparation is rotated at 33 rpm at room temperature for 30 minutes and placed at 37°C for 10–15 minutes.
4. Contents of the tube are transferred to a Wintrobe tube and centrifuged for 10 minutes.
5. Buffy coat smears are prepared, dried in the air, fixed in methanol and are stained with Romanowsky stain.

Note

- Some degree of trauma to the leukocytes is necessary for a successful preparation of LE cell.
- As anti-nuclear antibodies do not act upon healthy living leukocytes.
- A good method of achieving the necessary degree of trauma is to rotate the whole blood sample to which glass beads have been added.

Interpretation (Fig. 14.1)

- LE cells are seen at the edge of a smear
- 500 polymorphs are counted before a negative result is given.
- *Note:* LE cells must be differentiated from "tart cells" which are usually monocytes that have phagocytosed the nucleus of a lymphocyte.

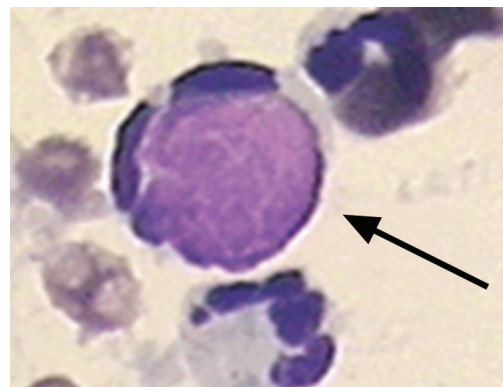


Fig. 14.1: LE cell (arrow)

Leukopoiesis

Definition: Process of generating white blood cells (leucocyte) from pluripotent hematopoietic stem cells of bone marrow.

There are two significant pathways:

- a. **Myelopoiesis** in which leukocytes in blood are derived from myeloid stem cells.
 - b. **Lymphopoiesis** in which leukocytes of the lymphatic system (lymphocytes) are generated from the lymphoid stem cells.
- From the early progenitor cell with lymphoid potential, there arises pro-NK cell, pro-B cell and pro-T cell which give rise to NK cell, B cell and T cell, respectively.
 - From CFU-mix (colony forming unit-mix population) arises myeloblast, monoblast, eosinophiloblast which give rise to neutrophils, monocyte, and eosinophil, respectively
 - From CFU-b/M/E arises basophiloblast, megakaryoblast and erythroblast which give rise to basophils, platelets and erythrocytes, respectively.

Leukemia

Leukemia is defined as cancer of blood cells.

- Bone marrow normally produces hematopoietic elements in mature form into the peripheral blood.
- When bone marrow produces increased number of precursor cells, there occurs an increased number of precursor cells in the blood too.
- As the blood cells are divided into myeloid and lymphoid precursors, there occurs myeloid and lymphoid leukemia.
- To be precise, precursors are further classified into varying populations.

Note: To learn more about the hematopoietic cell lines, kindly see chapter on leukopoiesis.

- Out of these precursor cell populations, if there occurs increase in number of blasts which make up more than 20% of the myeloid or lymphoid lineage cells, only then the term leukemia is used.
- So, in simple terms, if there are more than 20% of lymphoblasts in the bone marrow or peripheral blood, we report it as acute lymphoblastic leukemia (ALL).
- And, if there occurs more than 20% of myeloblasts in the bone marrow or peripheral blood, we report it as acute myeloid leukemia (AML).

- Leukemia can also be chronic and can involve both lymphoid and myeloid series of cells and are termed, respectively, as chronic lymphocytic leukemia (CLL) and chronic myeloid leukemia (CML).
- In CLL, there occurs increased proliferation of abnormal lymphoid cells.
- In CML, there occurs an increase in proliferation of precursors of myeloid lineage cells and the blast population remains less than 20%.

What is Responsible for Production of Abnormal Immature Cells?

- Due to genetic mutations in chromosomes of these patients, there occurs abnormal cell proliferation in the bone marrow.
- Ionizing radiation for treatment of tumors
- Accidental exposure of ionizing radiation can result in abnormal cellular proliferation in marrow
- Viruses, dyes and drugs are other responsible agents.

Clinical Features

- Patient presents with enlargement of lymph nodes, spleen, and liver.
- As the bone marrow function is not proper, hence there can be associated anemia, leukopenia and thrombocytopenia (i.e. pancytopenia).

- Because of these abnormalities in the normal cell production, these patients are predisposed for infections, fever, bleeding tendency and symptoms related to anemia.
- If leukemic cells spread to CSF, testes, brain, it is associated with poor prognosis.

Treatment

- For remission, different chemotherapeutic regimens are used, but invariably patients come back with the disease again after a gap of few months to years (called relapse).
- Bone marrow transplantation has shown promising results for curing these patients.

Osmotic Fragility Test

- Red cells are suspended in decreasing concentrations of hypotonic saline, to determine the ability of RBCs to withstand osmotic stress.
 - In hypotonic solutions, water enters red cells causing cellular swelling, followed by RBC lysis.
 - Normal RBCs are biconcave and disc shaped, have high surface area to volume ratio and therefore can increase their volume up to 70% before they are lysed.
 - With normal RBCs hemolysis starts at saline concentration of 0.5 gm/dl and is complete at 0.30 gm/dl.
 - In contrast, spherocytes have decreased surface area to volume ratio, and can undergo lysis earlier than normal RBCs.
 - In presence of spherocytes, red cells show beginning of hemolysis at 0.6 to 0.8 gm/dl, i.e. increased osmotic fragility.
 - Osmotic fragility is decreased in thalassemia, due to presence of target cells, since target cells have increased surface volume.
 - Sensitivity of the test can be increased by incubating the red cells at 37°C for 24 hours before performing the test (osmotic fragility test after incubation).
 - Na_2HPO_4 —13.6 gm
 - NaH_2PO_4 —2.4 gm
 - Distilled water—1000 ml
2. *Working solution:* Prepare 1 gm/dl buffered sodium chloride (NaCl) solution by mixing 10 ml of solution A and 90 ml of distilled water.
 3. Prepare working solutions in centrifuge tubes as follows (Figs 17.1 and 17.2):

S.no. of test tube	NaCl concentration	NaCl	Distilled water	Blood (sample)
1.	100%	5.0 ml	X	0.05 ml
2.	90%	4.5 ml	0.5 ml	0.05 ml
3.	75%	3.75 ml	1.25 ml	0.05 ml
4.	65%	3.25 ml	1.75 ml	0.05 ml
5.	60%	3.00 ml	2.00 ml	0.05 ml
6.	55%	2.75 ml	2.25 ml	0.05 ml
7.	50%	2.50 ml	2.50 ml	0.05 ml
8.	45%	2.25 ml	2.75 ml	0.05 ml
9.	35%	1.75 ml	3.25 ml	0.05 ml
10.	30%	1.50 ml	3.50 ml	0.05 ml
11.	20%	1.00 ml	4.00 ml	0.05 ml
12.	10%	0.50 ml	4.50 ml	0.05 ml
13.	0	0.00 ml	5.00 ml	0.05 ml

Procedure

1. Make a stock solution (A) of 10 gm/L
 - NaCl—90 gm
- Mix well. Keep at room temperature for 30 minutes, centrifuge.
- Read the results in a spectrometer at a wavelength of 540 nm.

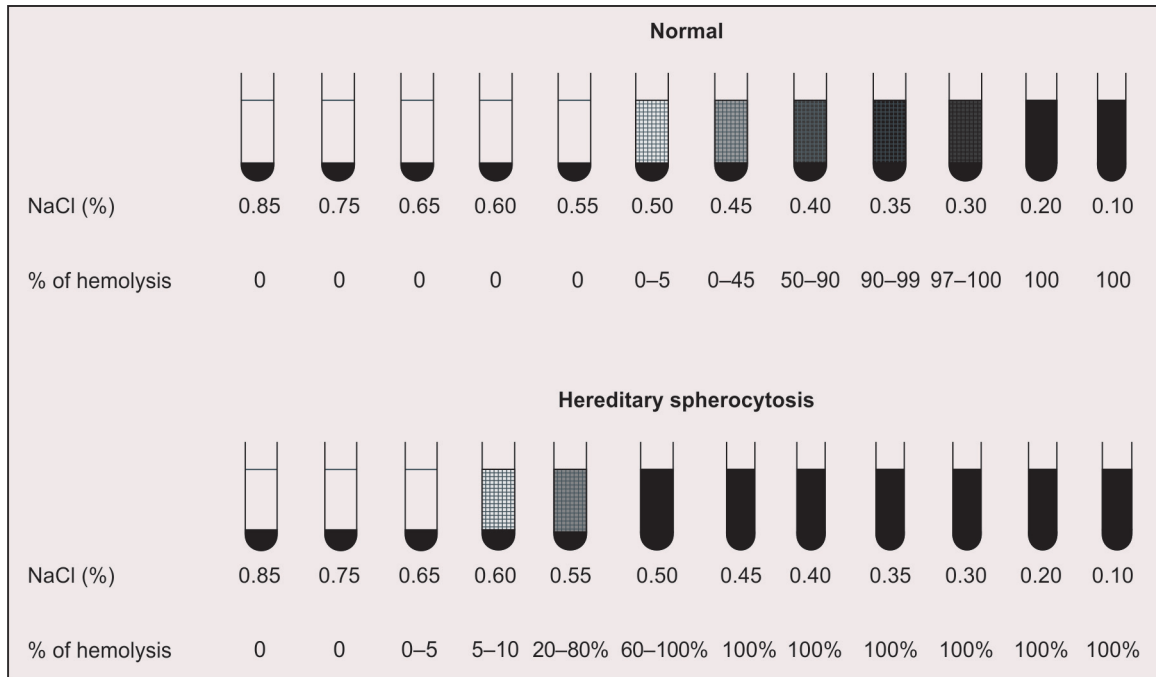


Fig. 17.1: Osmotic fragility is increased in hereditary spherocytosis as the hemolysis starts at lower concentration gradients of sodium chloride

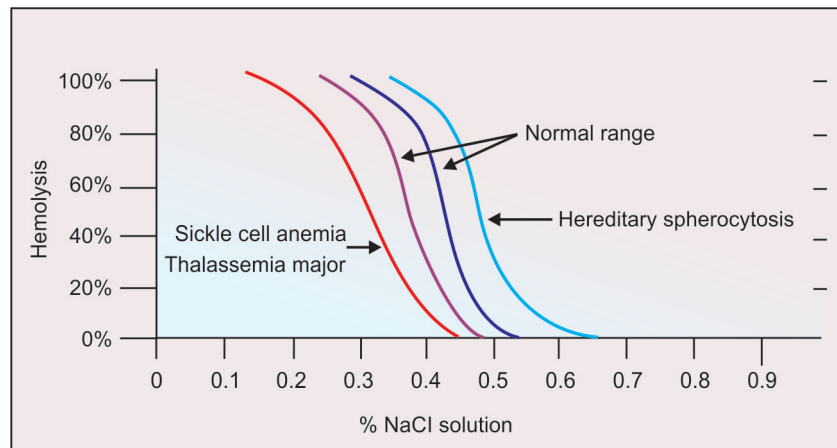


Fig. 17.2: Left shift to the normal range (reduced OF) is seen in sickle cell anemia and thalassemia. Right shift (increased OF) is seen in hereditary spherocytosis

Calculation

$$\% \text{ of hemolysis} = \frac{\text{Optical density of the sample}}{\text{Optical density of tube (showing complete hemolysis)}} \times 100$$

Packed Cell Volume (PCV)

- Volume occupied by RBCs when a sample of anticoagulated blood is centrifuged
- Also called hematocrit.

Uses of PCV

- Detection of the presence or absence of anemia or polycythemia.
- Estimation of red cell indices
- Estimation of hemoglobin using a simple calculation

PCV = Hemoglobin multiplied by ($\times$) 3

For example: If hemoglobin is 15 gm%, then PCV will be 45%.

Methods of Estimation

1. Wintrobe method
2. Microhematocrit method.

1. Wintrobe Method

Equipment

- a. Wintrobe tube (Fig. 18.1):
 - Tube is 110 mm in length
 - Tube has 100 markings on its surface, each marking at an interval of 1 mm.
 - Internal diameter of tube is 3 mm
- b. Pasteur pipette
 - It should reach the bottom of Wintrobe tube
- c. Centrifuge machine

Specimen: EDTA blood



Fig. 18.1: Wintrobe's tube

Methods

- Mix the anticoagulated blood sample thoroughly.
- Draw the blood in Pasteur pipette and introduce the pipette up to the bottom of the Wintrobe tube.
- Fill the tube from the bottom up to 100 mark
- Centrifuge it for 30 minutes

- Take the reading of the length of the column of red cells.
- Hematocrit/PCV is expressed as percentage

Interpretation (Fig. 18.2)

- After centrifugation, following zones are seen in Wintrobe tube from above downwards—plasma, buffy coat layer, and packed red cells.
- Length of the column of red blood cells indicates packed cell volume.
- In anemia, PCV is below the lower level of normal range.
- PCV is raised in dehydration, shock, burns, polycythemia.

Uses of buffy coat layer

- Buffy coat layer is a small grayish layer of WBCs and platelets, about 1 mm thick.
- Smears from the buffy coat layer are used for demonstration of lupus erythematosus (LE) cell, malarial parasite.

2. Microhematocrit Method

Equipment

- Microhematocrit centrifuge should provide a centrifugal force of 12000 g for 5 minutes.

- Capillary hematocrit tubes: Disposable glass tubes 75 mm in length and 1 mm in internal diameter.

- Tube sealant like plastic sealant or clay

- Microhematocrit reader or graph paper.

Methods

- Fill the capillary tube by applying its tip to the blood and the tube should be filled with $\frac{3}{4}$ of its length.
- Seal the other end of capillary tube with sealant
- Centrifuge these tubes
- Remove these tubes from the centrifuge machines and stand them upright.
- Tubes will show three layers from top to bottom: Plasma, buffy coat, column of red cells.

Note: Rules of 3 and 9

$$\text{PCV} = \text{hemoglobin (gm/dl)} \times 3$$

$$\text{PCV} = \text{red cell count (million/cu mm)} \times 9$$

Reference values of PCV

Adult males: 40–50%

Adult females: 38–45%

Adult females (pregnant): 36–42%

Newborns: 44–60%

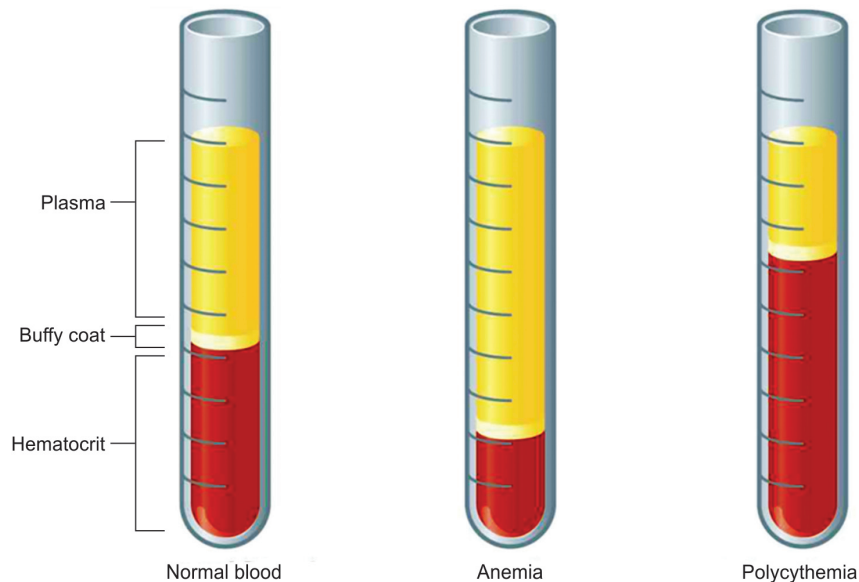


Fig. 18.2: Packed cell volume assessment

Phlebotomy

- Also called venipuncture
- Procedure of withdrawing blood from a vein.
- And the individual who performs this procedure is called phlebotomist.
- Phlebotomist should be either a doctor, nurse, medical laboratory scientist who are well-trained with the procedure.
- Phlebotomy can be diagnostic or therapeutic
- Diagnostic phlebotomy includes taking the blood samples for hematological, microbiological or biochemical studies.
- Therapeutic phlebotomy involves taking blood sample as a treatment protocol for hemochromatosis and polycythemia.
- Phlebotomy is done for collection of blood from donors in blood banks.
- Approach the patient in a friendly, calm manner and try to gain the patient's cooperation.
- Properly fill out appropriate requisition forms, indicating the tests ordered.
- Patient's condition should be noted on the lab requisition form, i.e. fasting, dietary restrictions, medications, timing, and medical treatment.
- Check for allergies to antiseptics, adhesives
- Position the patient
- Make the patient sit in a chair, lie down or sit up in a bed.
- Hyperextend the patient's arm
- Apply the tourniquet 3–4 inches above the selected puncture site.
- Do not place too tightly or leave on for more than 2 minutes.

Venipuncture Sites

Median cubital vein and cephalic veins are most commonly used.

Procedure for Vein Selection

- Palpate and trace the path of veins with the index finger.
- If superficial veins are not seen, it is advisable to force blood into the vein by massaging the arm from wrist to elbow.

Performance of a Venipuncture

- Identify the patient correctly
- The patient should make a fist without pumping the hand.
- Select the venipuncture site
- Prepare the patient's arm using an alcohol preparation.
- Cleanse in a circular fashion, beginning at the site and working outward and allow it to air dry.
- Needle should form a 15 to 30° angle with the surface of the arm.
- Insert the needle through the skin and into the lumen of the vein.

- Avoid trauma and excessive probing
- After the sampling is done, remove the tourniquet.
- Remove the needle from the patient's arm
- Press down on the gauze once the needle is out of the arm, applying adequate pressure in order to avoid hematoma formation.
- Dispose off the contaminated material in designated bags.
- Mix and label all appropriate tubes, in which the blood is taken to conduct tests.
- Make sure the samples should reach the laboratory as soon as possible.

Platelets

- Platelets are small, 1–3 microns in diameter, structures with irregular projections on their surface.
- Platelets are produced when the fragments of megakaryocytes are shed in the bone marrow.
- Lifespan of platelet is 7–10 days
- Normal count is 1.5–4.5 lakh/ microliter
- 2. **Secretion:** Release of their contents of coagulation factors.
- 3. **Aggregation:** Large numbers of platelets sticking to each other inside the wound thus forming a plug to stop blood flow through the wound.

Platelet contains two types of cytoplasmic granules

- α -Granule components*
 - Have adhesion molecule P-selectin on their membranes.
 - *Proteins involved in coagulation:* Fibrinogen, coagulation factor V, and vWF.
 - *Factors involved in wound healing:* Fibronectin, platelet factor-4, platelet-derived growth factor (PDGF), and transforming growth factor- β .
- Dense (or δ) granule components:* Adenosine diphosphate (ADP), adenosine triphosphate, ionized calcium, serotonin, and epinephrine.

Platelets Function

Platelets themselves serve as actual plugs which block the wound. This is achieved by aggregation of platelets at wound site, which blocks the wound and prevents bleeding. This is accomplished through the following steps:

1. **Adhesion:** Platelets sticking around the wound.

Thrombocytopenia

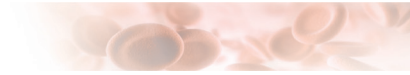
Defined as reduction in platelet count.

Causes

- Decreased production of platelets*
 - Infections: Measles, HIV
 - Vitamin B₁₂/folate deficiency
 - Leukemia, cancers, myelodysplastic syndromes.
- Decreased platelet survival*
 - Immune thrombocytopenic purpura (acute and chronic)
 - Systemic lupus erythematosus
 - B cell lymphomas
 - Drugs: Heparin, quinidine
 - Disseminated intravascular coagulation.

Megakaryopoiesis

- Process concerned with the production of platelets, which take place in bone marrow and result in the release of platelets into peripheral blood.
- Roughly, around $35,000 \pm 4300$ platelets/ml are made per day.



- Platelets are released into the blood due to the fragmentation of cytoplasm of megakaryocytes.

Maturational Sequence

Five morphologically identifiable stages of megakaryocytic cells in the bone marrow:

1. **Megakaryoblast:** 15–50 microns in size with blue cytoplasm and nuclei showing fine nuclear chromatin with 1–2 nucleoli.
2. **Pro-megakaryocyte:** 20–80 microns in size with the nucleus showing 2–3 nucleoli.
3. **Granular megakaryocyte:** Cell size varies from 30 to 90 microns, has abundant cytoplasm with reddish blue granules and contains numerous nuclei with coarse nuclear chromatin.
4. **Mature megakaryocyte**
 - Largest cell seen in the bone marrow with abundant cytoplasm.
 - Platelets start breaking up from the cytoplasm into the peripheral blood.
5. **Platelet:** Small fragments of the megakaryocyte cytoplasm and are of 1–4 microns in size.

Red Cell Indices

Red cell indices include

- Mean corpuscular volume (MCV)
- Mean cell hemoglobin concentration (MCHC)
- Mean cell hemoglobin (MCH)
- Red cell distribution width (RDW)

Uses of Red Cell Indices

- Classification of anemia into normocytic normochromic, microcytic hypochromic or macrocytic anemias.
- Differentiation of iron deficiency anemia (low MCV, MCH and MCHC) from thalassemia trait (low MCV and MCH with normal MCHC).

1. Mean Cell Volume

- Measure of average size of RBCs
- $$MCV = \frac{PCV\%}{\text{Red cell count in million per mm}^3} \times 100$$
- MCV is expressed in femtoliters or fl
- Normal MCV—80–100 fl

Note

- Mentzer's index is derived by dividing MCV with RBC count.

- Index value of less than 13—seen in thalassemia.
- Index value of more than 13—seen in iron deficiency anemia.

2. Mean Cell Hemoglobin (MCH)

- Amount of hemoglobin in a single red blood cell
- $$MCH = \frac{\text{Hemoglobin in gm}\%}{\text{Red cell in million per mm}^3} \times 100$$
- MCH is expressed in picograms or pg
- Normal MCH—27–32 pg

3. Mean Cell Hemoglobin Concentration

- Concentration of hemoglobin in 1 liter of packed red blood cells
- $$MCHC = \frac{\text{Hemoglobin in gm}\%}{PCV \text{ in } \%} \times 100$$
- Normal MCHC = 30–35 gm%

4. Red Cell Distribution Width (RDW)

- Measure of degree of variation in red cell size
- Low in beta-thalassemia trait, high in iron deficiency anemia and normal in anemia of chronic disease
- Normal RDW—11.5–14.5

Reticulocyte Count

Reticulocytes (Fig. 22.1)

- Reticulocytes are young RBCs containing remnants of RNA and ribosomes.
- Recognized by supravital stains which detect RNA in these cells.
- RNA appears as blue precipitating granules within RBCs.
- Supravital staining refers to staining of these cells in a living state.

Principle

Few drops of blood are incubated with methylene blue solution which stains granules of RNA in red cells.

Reagent

New methylene blue solution is prepared as follows:

- New methylene blue—1 gm
- Sodium citrate—0.6 gm
- Sodium chloride—0.7 gm
- Distilled water—100 ml

Reagent should be kept stored in a refrigerator at 2–6°C and filtered before use.

Other dyes used include: Brilliant cresyl blue and azure B

Sample: EDTA blood sample.

Methods

1. In a test tube, take 2–3 drops of filtered new methylene blue solution.

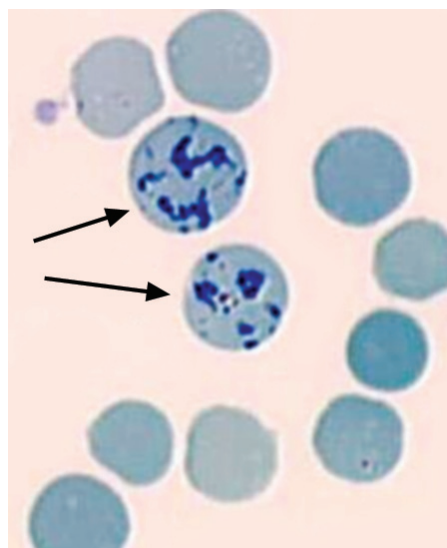


Fig. 22.1: Reticulocytes (arrows)

2. Add equal amount of blood and mix well
3. Keep the mixture at room temperature for 15 minutes.
4. Prepare a smear from the small drop of mixture prepared above.
5. Slide should be viewed under oil immersion lens in a microscope.
6. Reticulocytes show deep blue precipitates in the red blood cells.

Result

Number of reticulocyte is expressed as a percentage of red cells.

Reference Range

Reticulocyte percentage—0.5–2.5%

Increased reticulocyte count (reticulocytosis) is seen in

- a. Hemolytic anemia
- b. Blood loss
- c. Following treatment of nutritional anemia (like iron deficiency, folate deficiency, vitamin B₁₂ deficiency)
- d. Hemoglobinopathies, e.g. sickle cell anemia

Decreased reticulocyte count (reticulocytopenia) is seen in

- a. Aplastic anemia
- b. Bone marrow infiltration (leukemia, lymphoma, malignancy)
- c. Renal disease
- d. Anemia of chronic disease
- e. Alcoholism
- f. Ineffective erythropoiesis as seen in megaloblastic anemia, thalassemia.
- g. Following blood transfusion

Sickling Test

- Red blood cells, which are deprived of oxygen, containing HbS become sickle shaped.
- Reducing agent (to remove oxygen from red cells) is 2% sodium metabisulfite.
- Preparation is examined under the microscope after 30 minutes.
- If sickle cells are not seen, examine the slides again after 2 hours, followed by 24 hours.

Procedure

- Drop of capillary or anticoagulated venous blood is mixed on a glass slide with a drop of 2% sodium metabisulfite.
- A coverslip is placed over the mixture and sealed with petroleum jelly/paraffin wax.
- Test is reported negative, if the red cells remain round and positive if red cells become sickle shaped (crescent shaped with pointed ends) or holy-leaf shaped.

Note: It is necessary to perform hemoglobin electrophoresis for confirmation of HbS.

Total Leukocyte Count

- Refers to the number of white blood cells in 1 microliter of blood.
- *Methods of estimation:* Manual or microscopic method, automated method.
- *Purpose:* To detect increase or decrease in total number of white blood cells in the blood.
- c. Graduated pipette of 1 ml
- d. Pasteur pipette
- e. Test tube

Reagents

- Turk's fluid (WBC diluting fluid) consists of glacial acetic acid, which lyses red blood cells and gentian violet, which stains leukocyte nuclei deep violet.
- Composition of Turk's fluid: Glacial acetic acid (2 ml), gentian violet (1 ml), distilled water (100 ml).

Method

Add 0.1 ml of blood into 1.9 ml of diluting fluid, in order to get 1:20 dilution.

1. Manual Method

Principles

- Sample of whole blood is mixed with a diluent, which lyses red cells and stains nuclei of the white blood cells.
- White blood cells are counted in a Neubauer counting chamber under the microscope.

Equipment used

- Neubauer counting chamber*
 - Composed of ruled areas on the surface of the chamber.
 - Central large square is divided into 25 squares, each of which is divided into 16 small squares.
 - There are surrounding large four corner squares.
 - Four large corner squares are used for counting leukocytes.
 - Central large square is used for counting platelets and red blood cells.
- Pipette calibrated to deliver 20 microliters (0.02 ml, 20 cu mm)

2. Charging the Chamber

- Place the coverslip on the Neubauer's chamber.
- Draw some of the diluted blood in a Pasteur pipette.
- Hold the Pasteur pipette at an angle of 45° and place the tip between the coverslip and the chamber.
- The sample should cover the entire ruled area, and should not contain air bubbles.
- Allow 2 minutes for settling of cells.

3. Counting of Cells

- WBCs should be counted on the chamber

- Total cell count = Number of WBCs counted $\times 50$

Normal range: In adults: 4000–11000/ mm^3

Note: A bulb pipette should not be used for counting WBCs as it is not possible to obtain reliable mixing of blood and diluting fluid inside the bulb of the pipette.

RBC COUNT

- For RBC count, RBC pipette is used, which has a red dot.
- Diluting fluid used for RBC count is Hayem's fluid which is composed of mercuric chloride, sodium sulfate, sodium chloride, distilled water.
- Hayem's fluid results in RBC hemolysis.
- Aspirate the blood up to 0.5 mark of the pipette and the diluting fluid should be aspirated up to 101 mark. Hence, blood specimen is diluted in a ratio of 1:200 with the RBC diluting fluid.
- Hold the pipette horizontally, and roll it with both the hands between the finger and thumb.
- Hold the pipette at an angle of 45° and place the tip between the coverslip and the chamber.
- Allow the cells to settle down for 2 minutes
- Count the total number of cells in the red boxes as depicted in Fig. 24.1.
- After counting the cells, total number of RBCs are calculated as follows.
- N (total number of RBCs/ mm^3 of blood) = Number of RBCs counted on the chamber $\times 10000$

PLATELET COUNT

Diluting fluid: 1% ammonium oxalate

Principle: 1% ammonium oxalate lyses the red blood cells.

Procedure

- Take 950 microliters of diluting fluid in a clean, dry test tube.
- Add 50 microliters of anticoagulated blood sample and mix.

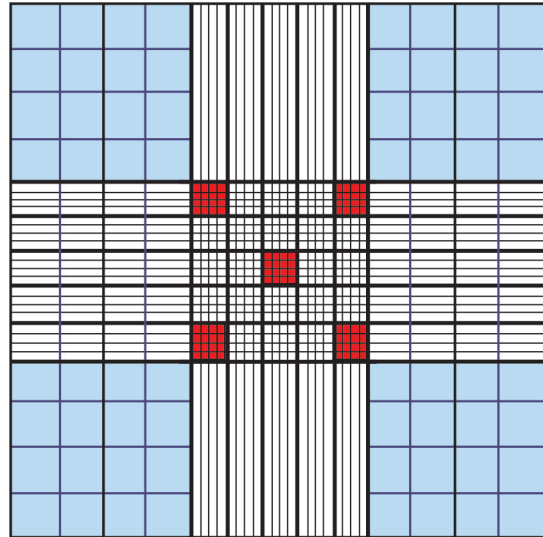


Fig. 24.1: Blue areas: WBCs are counted. Red areas: RBCs/platelets are counted

- Keep for 5 minutes at room temperature
- Mix and fill the chamber with the help of pipette.
- Keep the charged chamber in a moist petri-dish for 5 to 10 minutes and allow the platelets to settle down.
- Count the platelets in the same area as of RBC squares (n).
- Total number of platelets = $n \times 1000/\text{mm}^3$

EOSINOPHIL COUNT

Diluting fluid: Dungen's fluid

Composition: Eosin yellow—0.5 gm, 95% acetone—0.5 ml, 40% formalin—0.5 ml, distilled water—99 ml.

Principle

Blood is diluted with a diluting fluid, which stains the eosinophilic granules brightly and lyses RBCs and WBCs.

Procedure

- Take 950 microliters of diluting fluid in a clean, dry test tube.
- Add 50 microliters of anticoagulated blood sample and mix.

- Keep for 5 minutes at room temperature
- Mix and fill the chamber with the help of pipette.
- Keep the charged chamber in a moist petridish for 5 to 10 minutes.
- Count the eosinophils under low power (10x) in four corner squares with reduced light (N).
- Absolute eosinophil count = $N \times 50/\text{mm}^3$.
- Always clean the chamber before and after use.
- After taking blood in pipette clean the outer surface of the tip before diluting it in the diluting fluid.
- Always mix the dilution before filling the chamber.
- Avoid bubbles to come in the chamber
- Never overflow the chamber with dilution
- Change the coverslip, if it is dirty and scratched.
- Calculation must be done properly
- Clean the microscope before and after use.

Precautions while using Neubauer Counting Chamber

- Preparation of the diluting fluids must be proper.

White Blood Cells (WBCs)

- White blood cells are spherical cells, with a nucleus, and appear white in color as it lacks hemoglobin.
- *Five types of WBCs:* Granulocytes (neutrophils, eosinophils, basophils) and agranulocytes (lymphocytes, monocytes).

Granulocytes (Fig. 25.1)

a. Neutrophils

- 10–12 microns in diameter
- Nucleus with two to four lobes, connected by thin filaments
- Reddish purple, cytoplasmic granules
- *Function:* Phagocytosis and killing of infectious agent.

b. Basophils

- 10–12 microns in diameter
- Nucleus with two indistinct lobes, cytoplasmic granules stain blue-purple.
- Releases histamine which promotes inflammation and heparin which prevents clot formation.

c. Eosinophils

- 11–14 microns in diameter
- Bilobed nucleus, cytoplasmic granules look orange red or bright red.
- Releases chemical that reduces inflammation and attacks parasites.

d. Lymphocytes

- 6–14 microns in diameter

- Round nucleus
- Produces antibodies and other mediators for destroying microorganisms.
- Plays a role in allergic reactions, graft rejections, tumor control and regulation of immune system.

e. Monocytes

- 12–20 microns in diameter
- Nucleus appears round, kidney shaped or horseshoe shaped and has abundant cytoplasm.
- Phagocytic cell in blood and in tissues gets converted to macrophages, which phagocytose bacteria, dead cells and microorganisms.

Normal WBC counts: 4000–11000 cells/mm³

Differential white blood cell count

- Neutrophils: 40–75%
- Lymphocytes: 20–40%

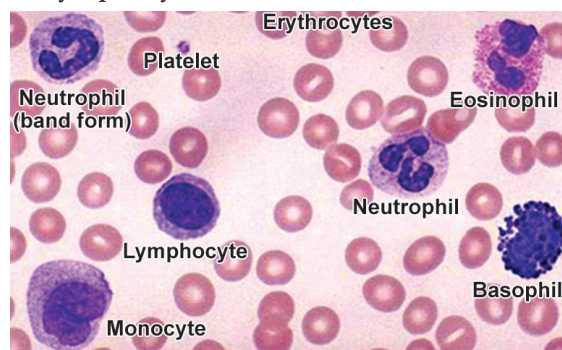


Fig. 25.1: White blood cells in peripheral smear

- Eosinophils: 1–6%
- Monocytes: 2–10%
- Basophils: 0–1%

Leukopenia: Low white cell count (leukopenia)

Neutropenia: Reduction in number of neutrophils in the blood.

Causes

- a. *Inadequate production:* Marrow suppression due to tumors, drugs (alkylating agents, antimetabolites) or granulomas.
- b. *Increased destruction:* SLE, splenomegaly, bacterial and fungal infections.

Lymphopenia: Reduction in lymphocyte count.

Causes: Human immunodeficiency virus (HIV) infection, corticosteroids, or cytotoxic

drugs, autoimmune disorders, malnutrition, acute viral infection.

Leukocytosis: Increase in number of white cells in the blood.

Causes: Chronic infection, myeloproliferative disorders (e.g. chronic myeloid leukemia).

Neutrophilic leukocytosis (increased neutrophil count): Acute bacterial infections.

Eosinophilic leukocytosis (increased eosinophil count): Allergic disorders, parasitic infestations, lymphomas, autoimmune disorders.

Basophilia (increased basophil count): Myeloproliferative disorders.

Monocytosis (increased monocyte count): Tuberculosis, endocarditis, malaria.

Lymphocytosis (increased lymphocyte count): Tuberculosis, viral infections.