Section I

Haematology

Exercise

1

Haemopoiesis

Formation of Blood Elements from Gestational Life to Adult Life

Cellular differentiation, proliferation and maturation of blood cells take place in the haematopoietic tissue, i.e. in the bone marrow (Fig. 1.1). Mature cells are released into the peripheral blood.

Development of haematopoiesis takes place at different places during gestational period and after birth and it is as follows:

1. Yolk sac—begins on 19th day of gestation and lasts up to 3 months.
2. Liver along with spleen, kidney, thymus, and lymph nodes—3rd month of gestation to 24 weeks.
3. Bone marrow—3rd trimester onwards and throughout life.

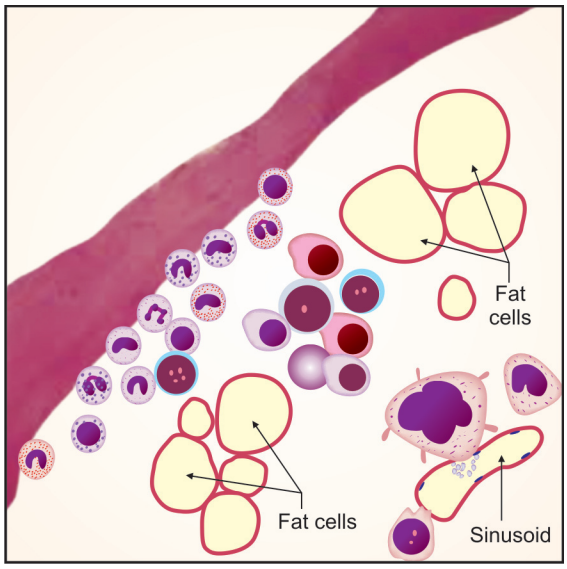


Fig. 1.1: Schematic diagram of topography of bone marrow cells of different series (paratrabecular area—myeloid series, near the sinusoids—megakaryocytic series, in between area—erythroid series)

HAEMATOPOIETIC MARROW ARCHITECTURE

Nucleated cells of RBC series constitute 25–30% of marrow cells and are produced near the sinusoids. Erythroblastic island is composed of erythoblasts in varying states of maturation. Least mature cells are towards the centre of the island and more mature cells towards the periphery.

Granulocytes are produced in the nests, close to the trabeculae. At the metamyelocyte stage they begin moving towards the sinusoids.

Lymphocytes are produced in lymphoid tissues (nodules) which are randomly dispersed throughout marrow. Lymphoid stem cells may leave the bone marrow and travel to thymus where they mature into T-lymphocytes. Some lymphocytes remain in bone marrow where they mature into B-lymphocytes.

Megakaryocytes lie adjacent to the endothelium of sinusoidal walls and discharge platelets directly into lumen of sinuses. Cytoplasmic processes of megakaryocyte penetrate the sinus wall and pinch off to form platelets.

ERYTHROID SERIES

It is an orderly process through which peripheral concentration of RBCs is maintained in a steady state.

Bone marrow maturation of normoblast occurs in orderly and well-defined sequence.

The process involves gradual decrease in cell size, together with condensation and eventual expulsion of nucleus.

As normoblasts mature, there is gradual increase in haemoglobin production. Normoblast generally spends 5–7 days in proliferating and maturing compartment of the marrow.

After maturation in the marrow the reticulocytes are released into the marrow sinuses and gain access to peripheral blood. It continues to mature in blood for 1 or 2 days.

Description of Erythroid Series Cells (Fig. 1.2)

Erythroblast (Normoblast/pronormoblast)

- 14–20 μ, round shaped, nucleus round
- Nucleus large occupies 4/5th of the cell and cytoplasm is 1/5th of the cell

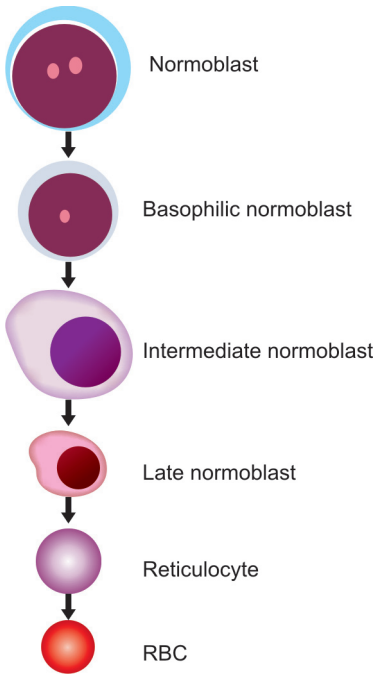


Fig. 1.2: Schematic diagram of eythroid series cells

- The cytoplasm is basophilic
- The nucleus has nucleoli
- Dividing cell.

Basophilic Erythroblast (Basophilic/Early normoblast)

- 12–15 μ
- Round-shaped
- Basophilic cytoplasm
- Nucleus-chromatin is dense
- Dividing cell.

Polychromatic Erythroblast (Intermediate normoblast)

- 12–14 μ
- Round-shaped
- Cytoplasm is polychromatic (purplish pink)
- Pink tint is because of haemoglobin
- Nucleus–chromatin clumped
- No division, cell develops by maturation.

Orthochromatic Erythroblast (Late normoblast)

- 12–14 μ
- Round-shaped
- Cytoplasm—more pinkish because of increased content of Hb
- Nucleus small and pyknotic with blue black colour
- The cell matures produce next cell.

Reticulocyte

- 8 μ
- Slightly larger than normal RBCs
- Biconcave discoid-shaped
- Cytoplasm–polychromatic, contains RNA material which can be stained with supravital stains
- Matures to RBC in 1–2 days.

Red Blood Cell

- 7.2 μ
- Biconcave, discoid-shaped
- Centre 1/3rd is pale, peripheral 2/3 coloured pinkish.

MYELOID SERIES (Fig. 1.3)

Myeloblast

- 15–20 μ, round-shaped
- Nucleus-round, occupies 4/5th of the cell and cytoplasm is 1/5th of the cell
- Nuclear chromatin less coarser than that of lymphoblast
- The cytoplasm is basophilic
- The nucleus has 4–5 nucleoli
- Dividing cell
- Sometimes Auer rod is found in the cytoplasm. It is purplish pink in colour.

Promyelocyte

- Nucleus-round
- Nuclear chromatin coarse
- Cytoplasm has primary granules which are dusty and purplish pink
- Nucleoli are few, 1–2 min number
- Other features are similar to myeloblast
- Dividing cell.

Myelocyte

- Nucleus is round
- Nuclear chromatin still coarser, no nucleoli
- Cytoplasm less basophilic and abundant
- Specific granules also appear in the cytoplasm. Depending upon these granules, the cells are called neutrophilic myelocyte, basophilic myelocyte and eosinophilic myelocyte.

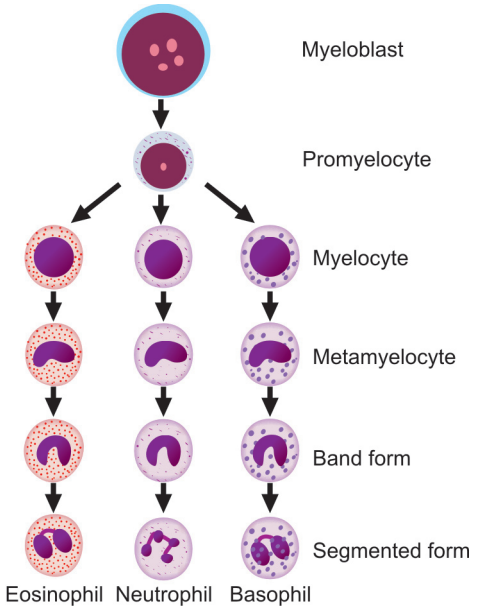


Fig. 1.3: Schematic diagram of myeloid series cells

Metamyelocyte

- Nucleus is kidney-shaped
- Cytoplasm is similar to earlier cell.

Band Form (Stab form)

- Nucleus more bent and attains U-shape. The degree of indentation is greater than 50% of the nuclear diameter
- Cytoplasm is similar to earlier cell
- These band forms mature to segmented forms.

Neutrophil (Polymorphonuclear leukocyte, segmented neutrophilic granulocyte)

- 12–14 μ
- Nucleus lobulated, has 2–5 lobes
- Cytoplasm has primary and secondary granules which are dusty and purplish pink coloured
- A sex chromatin (drumstick) may be present in some of the neutrophils attached to one of the lobes.

Eosinophil

- 14–16 μ
- Nucleus has two lobes (spectacular-shaped), cytoplasm has coarse granules which stain reddish or orange-coloured
- The granules do not overlap the nucleus.

Basophil

- 14–16 μ
- Nucleus has two lobes
- Cytoplasm has large round to oval deeply staining basophilic granules.

Note: Eosinophil and basophil are slightly larger than neutrophil.

LYMPHOID SERIES (Fig. 1.4)

Lymphoblast

- Nucleoli are 1–2
- Other features are similar to myeloblast.

Prolymphocyte

- Nucleoli are 0–1
- This cell divides and produces a large lymphocyte which matures to small lymphocyte.

Large Lymphocyte

- 12–16 μ
- Nucleus-round or indented
- Cytoplasm abundant, sky blue or pale blue coloured, a few azurophilic granules may be present.

Small Lymphocyte

- 6–10 μ
- Nucleus-round or indented
- Cytoplasm scanty and pale blue-coloured.

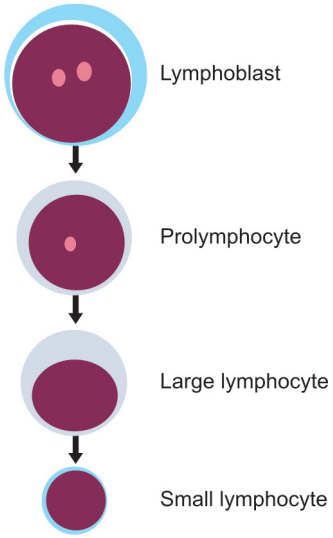


Fig. 1.4: Schematic diagram of lymphoid series cells

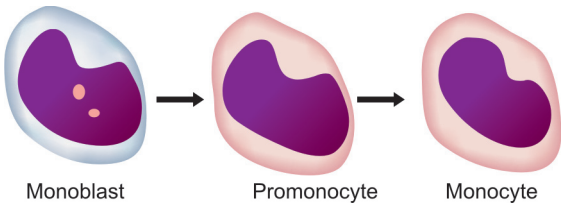


Fig. 1.5: Schematic diagram of monocyte series cells

MONOCYTE SERIES (Fig. 1.5)

Monoblast

- Nucleus-round or indented or convoluted
- Other features are similar to myeloblast.

Promonocyte

- Nucleus indented, can have clefts or convolutions
- Cytoplasm has azurophilic purplish pink granules
- Other features are similar to promyelocyte.

Monocyte

- 14–20 μ
- Nucleus lobulated, indented, kidney-shaped or has convolutions
- Nucleus has fine chromatin
- Cytoplasm grey blue, groundglass and abundant, fine azurophilic purplish pink
- Granules may be present sometimes cytoplasm has vacuoles.

MEGAKARYOCYTIC SERIES (Fig. 1.6)

Megakaryoblast

Nucleus and cytoplasmic features are similar to myeloblast.

Promegakaryocyte

- Nucleus is bigger than megakaryoblast
- Nucleus is lobulated because of endo-reduplication of nucleus
- Cytoplasm basophilic, stains light blue and has azurophilic purplish pink granules.

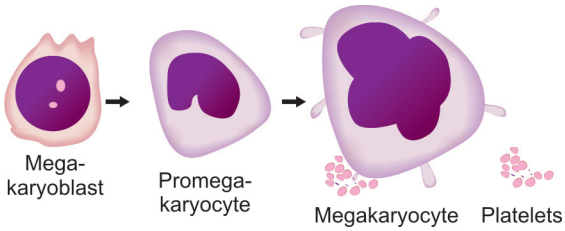


Fig. 1.6: Schematic diagram of megakaryocytic series cells

Megakaryocyte

- Largest cell in the bone marrow
- 30–90 μ
- Nucleus lobulated (4–16 lobes)
- Chromatin clumped
- Cytoplasm has azurophilic granules
- Platelets are formed by the protrusion of the pseudopodia of the megakaryocyte cytoplasm into the bone marrow sinusoids.

Platelets

- 1–4 μ
- Approximately 1/3 of an RBC size
- No nucleus
- Cytoplasm is light blue, has azurophilic purplish pink granules.

Exercise

2

Blood Collection

In investigating physiological functions and diseases related to blood, it is essential that the tests do not give misleading information. It is important to avoid faults in the specimen collection, storage and transport to the laboratory. Venous blood is preferred for most haematological investigations. Capillary blood is usually restricted to children; however for certain investigations, sample of blood needed is less and this blood can be used. Different sources, their techniques of obtaining and uses are as follows:

Sources

i. Capillary or peripheral blood
ii. Venous blood

i. *Capillary or Peripheral Blood*

When small quantity of blood is needed, this blood is preferred.

Sites

a. Ear lobe (free margin).
b. Tips of fingers—palmar surface, usually ring finger of left hand is used.
c. In infants great toe or heel—medial or lateral portions of plantar surface is chosen.

Skin which is oedematous, congested, cold and cyanotic should not be used.

Equipment

1. Gauze pads.
2. 70% alcohol.
3. Lancet (disposable or reusable).

Procedure

- The site chosen for collecting blood is cleaned with 70% alcohol which acts as disinfectant and also removes the dirt and epithelial cells.
- Allow it to dry.
- Give a firm quick stab with a sterile lancet.
- Ideal depth is 1 to 3 mm.
- Allow the blood to collect at the puncture site. Do not squeeze, as with squeezing tissue juices will contaminate and dilute the blood.
- Blood collects in the form of a drop.
- Wipe away first drop, as it may be contaminated with tissue juices.
- Allow free flow of blood.
- Collect the blood for necessary investigations.
- To stop the flow of blood, apply light pressure with gauze pad after necessary amount of blood is collected.

Uses

Capillary blood is used for the following investigations:

1. Cell counts—total WBC count, RBC count, absolute eosinophil count.
2. Peripheral smear.
3. Hb% estimation.
4. Micro ESR.

Note: Before puncturing immerse the heel in warm water or apply hot water compression. Otherwise, values may be significantly higher than the venous blood.

ii. *Venous Blood*

This blood is used when many tests are to be done and when quantity of blood required is more.

Site: Median cubital vein

Equipment

Syringe: Syringes of 2 ml, 5 ml or 10 ml capacity depending on the quantity of blood needed are to be used. Disposable plastic syringes are used. The gauge of the needle chosen depends upon the amount of blood needed; usually 21 or 22 gauge needle can be used. The length of the needle used is shorter for superficial veins (Fig. 1.7). If deep veins are selected the needle should be longer. The needle tip should be sharp. If blunt, it may cause trauma and pain to the patient.

Procedure

- Assure the patient with a few good words.
- Make the patient to lie down or give him/her comfortable sitting position.
- Do not make the patient to stand or make him/her to sit on high stool, as there are chances of fainting.
- Arm, which is to be venipunctured should be firmly supported.
- Inspect and evaluate the veins.

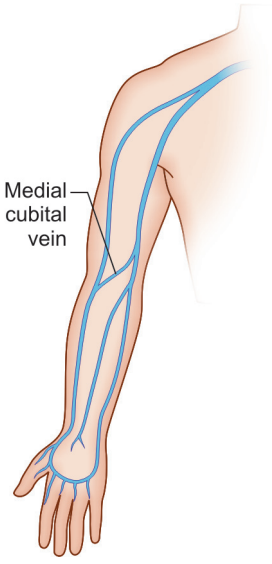


Fig. 1.7: Superficial veins of upper limb

- Apply the tourniquet above the vein (lower part of arm), which makes the vein more prominent. The pressure should not be maintained for longer time than necessary as it produces haemoconcentration.

If tourniquet is not used, ask the patient to close the fist firmly for some time. Another person can be asked to apply pressure over the vein. These steps are meant to make the vein prominent.

- Clean the site with 70% alcohol.
- Fix the vein in position by supporting the patient's forearm. Instruct the patient to hold the pad. Request him/her to raise the outstretched arm for a few minutes.
- Hold the syringe between the thumb and last 3 fingers of right hand, resting the back of these fingers on the patient's arm.
- Rest the free index finger against the hub of the needle and this serves as a guide.
- Push the needle into the prominent vein with a single direct puncture of skin and vein.
- This is known as one step procedure which is less painful.
- Entrance into the vein is followed by appearance of blood into the hub.
- After the desired amount of blood is obtained, withdraw the syringe and the needle.
- Apply gentle pressure to the puncture with dry cotton or gauze.
- About 5 to10 ml of blood can be obtained for different investigations.
- The blood in the syringe is used for investigations directly or it can be maintained in fluid state by putting it in an anticoagulant.

Differences between Capillary Blood and Venous Blood

Venous blood and the capillary blood are not the same; even if the latter is freely flowing as it is more nearly arteriolar in origin. The packed cell volume (PCV), red blood cell count (RBC count) and haemoglobin (Hb) of capillary blood are slightly higher than those of venous blood. The total leukocyte count (TLC/TC), neutrophil count and monocyte count are higher especially among children.

Exercise

3

Anticoagulants

Different kinds of anticoagulants are available for various purposes. Following are the commonly used anticoagulants in the haematology laboratory. These anticoagulants can be prepared in the laboratory or recently plain and containing different anticoagulant vacutainers are available commercially (Fig. 1.8).

i. Double oxalates—widely used
ii. EDTA—widely used
iii. Tri-sodium citrate—used in coagulation studies
iv. Heparin

Action of Anticoagulants

Oxalates prevent clotting by precipitating calcium (Ca) ions in the plasma. Sodium citrate and EDTA convert Ca ions into unionized form. Heparin acts as antithrombin and prevents formation of thrombin.

i. *Double Oxalates (Wintrobe's salt/mixture)*

Ammonium oxalate	2.4 g
Potassium oxalate	1.6 g
Distilled water	100 ml

Three parts of ammonium oxalate and 2 parts of potassium oxalate are used to balance the swelling effect of ammonium oxalate and the shrinkage effect of potassium oxalate. About 0.2 ml of solution (8 mg of chemicals) is used to prevent clotting of 4 ml of blood. The bottles with the solution are heated in an incubator at 60 to 80°C for one hour.

Use

Used for Hb%, cell counts and ESR.

Disadvantages

1. WBC's morphology is not preserved well, so the double oxalate anticoagulants are not to be used for peripheral smear preparation.
2. As it is toxic, it is not used for blood transfusion.

ii. *Ethylenediaminetetra-acetic (EDTA) Acid*

It is also known as versene. Disodium and dipotassium salts are preferred by International Council for Standardization in Haematology (ICSH). Calcium is converted to unionised form and forms a soluble complex. It is a powerful anticoagulant.

Fig. 1.8: Vacutainers

Dipotassium salt is easily soluble (1650 g/L) and hence preferred over to disodium salt (108 g/L) which is less soluble.

4 g/100 ml solution is prepared. About 0.1 ml (4 mg) of EDTA is put in vials and evaporated. 4 mg EDTA/2 ml of blood or 2 mg EDTA/ml of blood is used.

Uses

1. Gives good cellular morphology.
2. Clumping of platelets is prevented and hence, it is preferred for platelet counts.
3. Used for Hb% and cell counts.

Disadvantages

1. Platelets swell and disintegrate giving high platelet count.
2. RBCs and leukocytes show shrinkage and degeneration.
3. Excess EDTA gives decreased PCV and increased MCHC and Hb%.
4. Not suitable for coagulation studies.

iii. *Tri-sodium Citrate*

It binds calcium. One part of sodium-citrate to 9 part of blood is used for coagulation studies. About 0.4 ml of 3.8 g/dl of tri-sodium citrate is added to 1.6 ml of blood for ESR determination in Westergren's method.

Disadvantages

It is in liquid form. It dilutes the blood and hence it is not suitable for estimation of Hb% and cell counts.

iv. *Heparin*

Heparin neutralizes the thrombin, thus inhibits coagulation. About 15–20 IU heparin/ml of blood is used.

Uses

1. Best anticoagulant for osmotic fragility.
2. Size alteration of RBCs is nil, lysis is less.
3. Used for open-heart surgeries and emergency determination of blood sugar, urea and electrolytes.

Disadvantages

1. It is expensive.
2. Unsuitable for counts and smears, prevents coagulation for a limited period only.
3. Gives blue tint background, hence not suitable for peripheral smear.

Exercise

4

Peripheral Smear (Blood Film) Preparation and Staining

Peripheral smear (blood film) should be made on clean glass slides. Smears made on coverglasses are unsuitable for modern laboratory practice. Smear can be made by two slide manual method or by means of an automated slide spreader. Romanowsky stains are routinely employed for staining these peripheral smears and satisfactory results are obtained.

Two-Slide Method (Fig. 1.9)

- Take a slide and a spreader. The slide should be clean and free from dust, lint and grease. The spreader should be clean slide with sharp and even edges; sometimes the corners of the spreader are cut; by doing so we get a smear of lesser width than the width of a slide.
- A drop of fresh blood is taken either from the finger prick or sole in case of infants or from venous blood.
- The drop should be of moderate size.
- The drop is placed at a point three-fourth inch away from one end of the slide.
- Then the spreader is held between the thumb and the forefinger of right hand and then the drop of blood is touched with one end of it.
- Thus, the drop spreads along the edge of the spreader.
- Push the spreader forward immediately with an even and moderate spread, such that the drop spreads into a moderately thin smear.
- Air dry the smear either by waving the slide in air or by holding the slide near a fan or in front of a tube light.
- A good smear should be 3 cm in length. It should not cover the entire surface of the slide. It should have even and smooth appearance. Further it should be free from ridges, waves and windows (holes). The tail should not have any fringes (ragged ends).
- The smear has head, body and tail (Fig. 1.10). The beginning part which is slightly thicker is called head. The cells are overlapped here. The middle part is the body part; the cells are evenly spread in this region. The tapered part is called the tail. The smear is studied mainly in the body part.

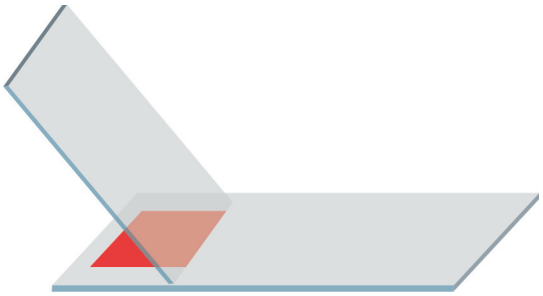


Fig. 1.9: Preparation of peripheral smear

Fig. 1.10: Schematic diagram of different parts of peripheral smear. A. Head; B. Body; C. Tail

- The slide is labelled by marking with lead pencil on the head region.
- Once the smear is prepared, it has to be fixed.
- Fixing is done by keeping the slide in methyl alcohol for 1 to 2 minutes. Even 1 to 2 dips are sufficient. This prevents crenation and artifacts of the cells.
- If the staining is done immediately with Wright's stain, there is no need of fixation. But if delay is expected, then first it has to be fixed, and later stained.

Staining of Peripheral Smear (Fig. 1.11)

Commonly employed Romanowsky stains are: Wright's stain and Leishman's stain.

Procedure

- Place the slides on a stain rack.
- Put sufficient Wright's stain on the smear. If the stain is less, it evaporates and leaves some stain particles on the smear.
- Wait for 2 minutes. But the timing varies depending on the freshness of the stain. If the stain is old and prepared long back, it may need more time (2½ to 4 minutes).
- Add equal quantity of buffer.
- Blow gently (this step can be avoided).
- Wait for 7 minutes.
- Discard the stain and the buffer.
- The back of the slide is cleaned with gauze or wiped with a blotting paper.
- Excess water is drained off.
- Stained smear is then dried by leaving in a slanting position or by blotting gently with a filter paper.

Note: Instead of buffer, tap water also can be used; but the pH of the water should be around 6.4; a well-stained smear should look pink or purplish pink.

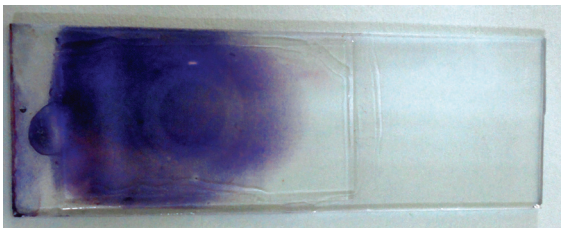


Fig. 1.11: Stained peripheral smear

Causes of Understaining

- More acidic pH of buffer or water
- If the staining period is shorter
- Too long wash
- Mounting the coverslip before the stained smear is dried.

In such smears the RBCs look red or orange. The nucleus and basophilic granules are not properly stained. The granules of the eosinophil are brilliant red. As the nucleus is not stained, the WBCs cannot be identified properly.

Causes of Overstaining

- More alkaline pH
- If the staining period is too long
- If washing time is too short or inadequate washing
- Thick films.

In such smears the RBCs are green or blue. The granules and nuclei are stained too dark even the granules of eosinophils stained blue grey.

Buffer Used

Refer to the topic on buffer.

Wright's Stain

Refer to Romanowsky stains.

Exercise

5

Haemoglobin (Hb) Estimation

The haemoglobin concentration can be estimated by various methods by measurement of colour, its power of combining with oxygen or carbon monoxide or by its iron content.

Acid Haematin Method (Sahli's method)

This is one of the colourimetric methods. Haemoglobin is converted into acid haematin by dilute HCl into brownish yellow coloured solution. This colour of the solution is matched with the colour comparator of Sahli's haemoglobinometer (Figs 1.12a and b).

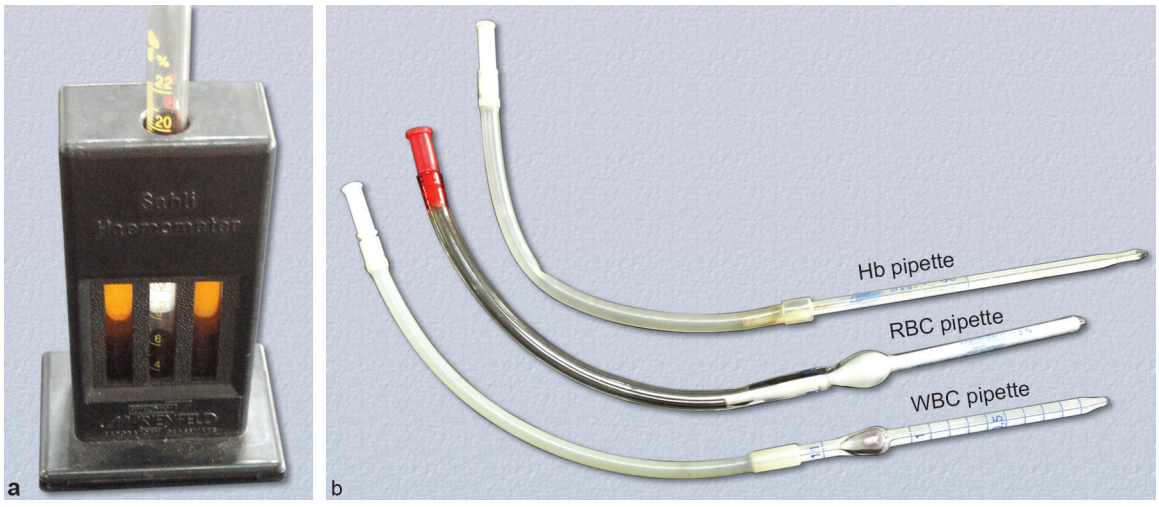
Blood used: Direct or anticoagulated blood.

Procedure: N/10 HCl is taken up to '20' mark of the graduated tube. About 20 cc of blood is taken in the haemoglobin pipette. The tip of the pipette is wiped with blotting paper. The blood is added to the HCl in the dilution tube. Wait for 10 minutes, and then go on adding distilled water till the colour matches with the colour comparator of Sahli's haemoglobinometer.

Haemoglobin is expressed in g/dl.

Other Methods

1. In colourimetric methods:
   a. Alkali haematin method
   b. Cynmeth haemoglobin method
   c. Tallquist method.



Figs 1.12a and b: (a) Haemoglobin estimation by Sahli's method; (b) Haemoglobin pipette with RBC and WBC pipettes

2. Specific gravity method.
3. Gasometric method.
4. Chemical method.

Normal Range

Men	13.5 to 15.5 g/dl
Women	12.5 to 13.5 g/dl
Newborn	16 to 18 g/dl
10 to 12 years of age	12 to 13 g/dl

Sources of Error in Sahli's Method

1. Non-haemoglobin substances like proteins and lipids in the plasma influence the colour of the blood that is diluted with acid.
2. About 2–12% of haemoglobin (sulphaemoglobin, methaemoglobin and carboxyhaemoglobin) is not converted into acid haematin.
3. Time has to be determined and every time the observation has to be made at the same interval.
4. Matching with glass standard may introduce some sources of error.
5. Variation from operator to operator in matching the colour is also possible.
6. The other possible errors are:
   - Errors of the sample
   - Errors of the equipment
   - Errors of pipette calibration
   - Unclean and wet pipette.

Cyanmethaemoglobin Method (Fig. 1.13)

Equipment

- Photoelectric colourimeter or photometer
- Drabkin's solution
- Haemoglobin pipette.

Composition of Drabkin's solution

Sodium bicarbonate (Sodium hydrogen carbonate)	1.0 g
Potassium cyanide	0.05 g
Potassium ferricyanide	0.20 g
Distilled water	1000.0 ml

Fig. 1.13: Photocolourimeter for haemoglobin estimation by cyanmethaemoglobin method

Principle

Potassium ferricyanide converts haemoglobin iron from ferrous to ferric state and forms methaemoglobin. This combines with potassium cyanide to produce cyanmethaemoglobin which is stable.

Cyanmethaemoglobin (HiCN) standards are commercially available. The OD of this is measured at 540 nm (green filter) which corresponds to 15 g/dl of haemoglobin.

Procedure

Take 5 ml of Drabkin's solution, mix 20 cc (0.02 ml) of blood and wait for 5 min. Read the absorbance of test by setting blank to 100%.

$$\text{Hb g/dl} = (\text{OD test}/\text{OD STD}) \times 15$$

Draw a graph by plotting OD on Y-axis and concentration on Hb, i.e. 5 g, 10.0 g, 15.0 g on X-axis. A straight line, passing through the origin, agrees with Beer's law. This graph can be used as a standard graph for Hb determination.

Principle of Photocolourimeter

A 6V, 3W lamp fed from IC stabilized power supply forms source of light. The light passes through the filter, then onto test tube containing the sample solution. After this, light falls on the photocell which is sensitive.

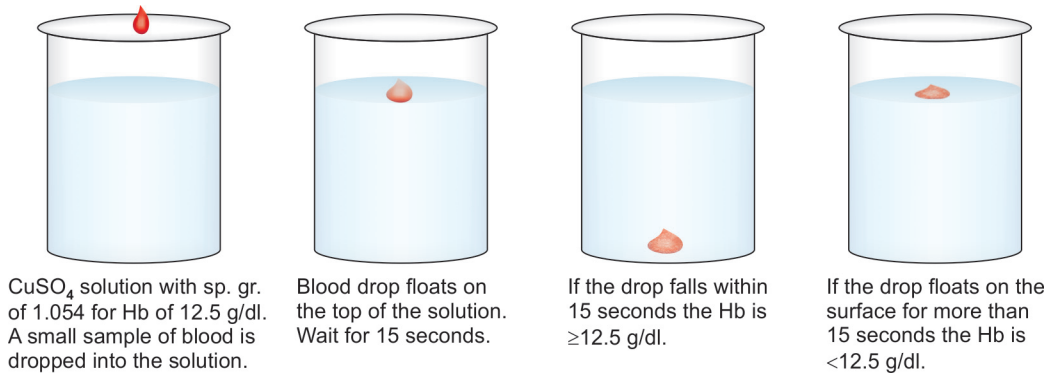


Fig. 1.14: Haemoglobin estimation by specific gravity method

Then the readings are read as log DPM. The DPM displays optical density from 0.00 to 1.99.

The working of photometer is based on Beer's or Lambert's law.

Beer's law states that optical density is directly proportional to the concentration of solution.

Lambert's law states that OD of coloured solution is directly proportional to the path of light (diameter of cuvette).

Tallquist Method

This is a colourimetric method. A drop of blood is put on a filter paper. The resulting colour is compared with the Tallquist plate; but this is not a reliable method.

Haldane's Method

In this method, a known volume of blood is converted into carboxyhaemoglobin by allowing it to act with carbon monoxide gas. The cherry red colour of carboxyhaemoglobin thus formed is compared with a standard tube of the same size containing carboxy-haemoglobin.

Specific Gravity Method (Fig. 1.14)

This method is used in blood donation camps. A drop of anticoagulated blood is dropped into copper sulphate solution of known specific gravity. In a few seconds (15–20 seconds), the drop begins to rise or continues to fall (rises if specific gravity of blood is less and falls if specific gravity is more). The accuracy depends upon the number of copper sulphate solutions of different specific gravity used.

The specific gravity of blood ranges from 1.048 to 1.066; the average for men is 1.057, and for women is 1.053. The values are lower by 0.003 in the afternoon and after meals. But after exercise and during night, the same values are higher. In cases of anaemias the specific gravity is less than the normal range.

Gasometric Method (By using von Slyke's apparatus)

By saturating the blood with oxygen first, then driving off the oxygen and collecting it separately, one can calculate the amount of haemoglobin. One gram of haemoglobin binds to 1.34 ml of oxygen.

Exercise

6

Cell Counts

NEUBAUER COUNTING CHAMBER
(Figs 1.15 and 1.16)

Various cell counts are being done using counting chamber. The visibility of the ruling in the chamber is important for the accuracy of the counts. The Neubauer or improved ruling is recommended.

- Neubauer counting chamber is made of a thick glass slide. It has H-shaped grooves. On either side of the horizontal gutter, there are two counting chambers.
- The total area of each chambers is 9 sq. mm (3 × 3 mm) and the depth is 0.1 mm. The area of the chamber is divided into 9 squares. The four corner squares are divided into 16 squares which are used for absolute eosinophil count and total leukocyte count. The central square is divided into 25 small squares. Each of these squares is again divided into 16 smaller squares.
- The central square is usually used for platelet count, whereas, five of the small squares (secondary squares) in the central square are used for RBC count.
- The coverslips used for Neubauer chamber should be smooth with even surface. The thickness is 0.3 to 0.5 mm. The sizes are 16 × 22 mm and 22 × 23 mm.

Fig. 1.15: Neubauer counting chamber with RBC and WBC pipettes in haemocytometer box

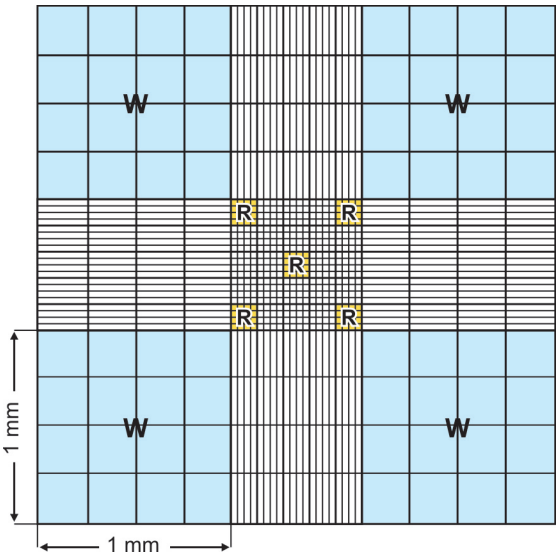


Fig. 1.16: Diagram of Neubauer counting chamber with markings W—WBC count, R—RBC count

Note:

- In improved Neubauer counting chamber, the 9 large squares are separated by solid black lines.
- Other counting chambers like Fuchus-Rosenthal and Speirs-Levy haemocytometers are with little modifications.

PLATELET COUNT

Reagents and Equipment

1. Counting chamber.
2. Anticoagulated blood (EDTA is the preferred anticoagulant).
3. RBC pipette.
4. Platelet diluting fluid (Rees-Ecker's fluid).

Composition of Rees-Ecker's Fluid

Brilliant cresyl blue	0.1 g
Sodium citrate	3.8 g
Neutral formaldehyde, 40%	0.2 ml
Distilled water to make	100 ml

Store in a refrigerator and filter before use.

Different diluent fluids like ammonium oxalate and formal-citrate red cell diluent fluids also can be used.

Procedure

Blood is drawn up to '1' mark of RBC pipette and diluting fluid up to '101' mark: these are properly mixed (Fig. 1.17). Wait for sometime, discard the first a few drops and then charge the chamber. It is preferable to place the chamber in a Petri dish containing wet cotton or filter paper to prevent evaporation. Allow some time for the cells to settle down.

The platelets appear as round to oval in structure. These should be differentiated from the debris, dust, etc. which are refractile. Count the platelets in the central large square (all the 25 small squares in the central large square).

Calculations

$$\frac{n \times \text{dilution factor}}{\text{Volume of chamber counted}}$$

$$= \frac{n \times 100}{0.1 \times 1 \times 1}$$

$$= n \times 1000 \text{ cells/cmm}$$

(n: Number of platelets counted)

Normal range: 1.5 to 4 lakh cells/cmm

RBC COUNT

Reagents and Instruments

1. RBC pipette.
2. Counting chamber.
3. RBC diluting fluid.

Composition of Hayem's Fluid

Mercuric chloride	2.5 g (to provide stability)
Sodium chloride	5.0 g (for isotonicity)
Sodium sulphate	25.0 g (to prevent rouleux)
Distilled water	1 litre (solvent)

Dissolve all the chemicals in distilled water and filter several times. Prepare a sufficient quantity to last only for 2–3 weeks, since deterioration may occur beyond this time, although Hayem's fluid remains fairly stable.

Procedure

Draw blood up to '0.5' mark of RBC pipette. Blood adhering to the tip is to be wiped off. Draw diluting fluid up to '101' mark. The pipette is shaken on the palms for about 30 seconds to facilitate mixing. Discard the first a few drops. Charge the chamber and wait for the cells to settle down. The chamber is visualised in low power and five of the 25 small squares of central large square are to be counted.

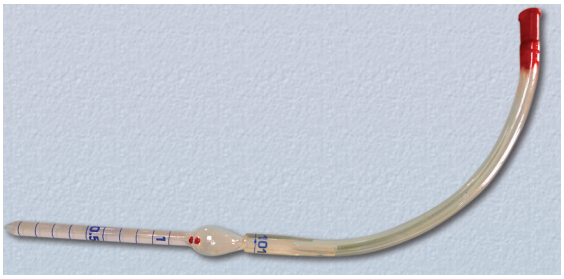


Fig. 1.17: RBC pipette

Calculation

$$\frac{n \times 200}{0.2 \times 0.2 \times 0.1 \times 5}$$

$= n \times 10{,}000$ cells/cmm

(n: Number of RBCs counted)

Other diluting fluids

Gower's solution

Sodium sulphate	62.5 g
Glacial acetic acid	167 ml
Distilled water	to make 1 litre

Dacie's solution

3% tri-sodium citrate	99 ml
Conc. formalin	1 ml

TOTAL LEUKOCYTE COUNT

The total leukocyte count (TLC) is meant to determine the number of leukocytes per µl (cmm) of the blood.

Equipment

1. WBC pipette (Fig. 1.18)—It has a stem and a mixing chamber
   Markings on the pipette—0.5, 1 and 11
   The volume of the mixing chamber is 20 times of the blood drawn up to 0.5 mark.
2. WBC diluting fluid (Turk's fluid):

Glacial acetic acid	2 ml
1% aqueous solution of Gentian violet	1 ml
Distilled water to make	100 ml

   The solution is stable at room temperature. A pinch of thymol may be added as preservative.
3. Counting chamber.
4. Blood-anticoagulated or fresh blood could be used.

Procedure

Blood is drawn up to '0.5' mark of the WBC pipette. Wipe off the blood sticking at the tip. Draw diluting fluid up to '11' mark. Mix the contents well between the middle finger and thumb. Wait for 5 minutes. Discard the first few drops. Charge the chamber. Count the WBCs in the corner 4 squares of the chamber—'n' number of cells. Do not count the WBC touching the dividing lines to the right and below, but include the cells touching the dividing lines to the left and above.

Calculations

$n \times$ dilution factor/volume of squares counted, 'n' being number of cells counted

$= n \times 20/0.4$

$= n \times 50$ cells/cmm

WBC count is expressed as … cells/cmm

Normal Values of Total Leukocyte Count

Adults	4000–11000 cells/cmm
At birth	10000–25000 cells/cmm
1–3 years	6000–18000 cells/cmm
4–7 years	6000–18000 cells/cmm
8–12 years	4500–13500 cells/cmm

Physiological Variations

- TLC is higher among females than among males. In females, TLC falls down after menopause.
- Oral contraceptive pills are reported to raise the TLC.
- *Diurnal variation:* Count is less in the morning and reaches maximum by afternoon.
- Physical exercise increases TLC up to 30,000 cells/cmm; the reason is that during

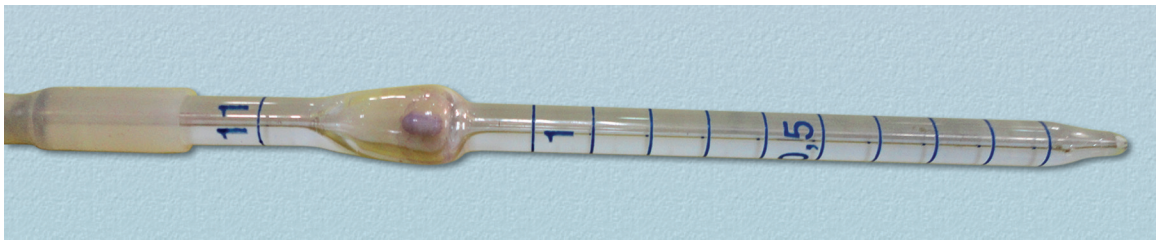


Fig. 1.18: WBC pipette

exercise splenic flow is reduced preventing sequestration of the cells. A large number of neutrophis, lymphocytes and monocytes enter the circulation during such exercise.

- Higher counts are observed with high temperature, severe pain, emotion, smoking, adrenaline administration and during pregnancy. Counts return to normal a week after delivery.
- At birth, the counts are high and gradually drop thereafter.

True Leukocyte Count/ Corrected Leukocyte Count

When the nucleated red cells are counted, they cannot be distinguished from leukocytes and if the number of nucleated red blood cells (NRBC) is higher in the stained smear, a correction is made according to the following formula:

True leukocyte count = (TC × 100)/(100 + NRBC)

(NRBC: Number of nucleated red cells, counted in differential count per 100 leukocytes)

Exercise

7

Red Cell Indices

Mean Corpuscular Volume (MCV)

Indicates volume of red cells. It is expressed in femtolitres (fl).

$$\text{MCV} = \frac{\text{Hematocrit} \times 10}{\text{RBC count in millions}}$$

Normal range 80–98 fl: Normocytes

< 80 fl: Microcytes

> 100 fl: Macrocytes

Mean Corpuscular Haemoglobin (MCH)

MCH indicates amount of Hb per red cell. It is expressed in pg (picograms)

$$\text{MCH} = \frac{\text{Hb (g/dl)} \times 10}{\text{RBC count in millions}}$$

Normal range: 26–34 pg

Less than 26 pg: Decreased MCH

Seen in microcytic hypochromic anaemias

More than 34 pg: Increased MCH

Seen in macrocytic anaemia

Mean Corpuscular Haemoglobin Concentration (MCHC)

MCHC denotes average concentration of haemoglobin in the red cells.

$$\text{MCHC} = \frac{\text{Hb (g/dl)} \times 100}{\text{Haematocrit (\%)}}$$

MCHC is expressed in g/dl

Normal range: 31–37 g/dl

<31 g/dl—hypochromic

>37 g/dl—hyperchromic (spherocytes)

Red Cell Distribution Width (RDW)

Provides an assessment on variation in red cell volume.

In early iron deficiency anaemia RDW is increased with normal MCV.

In established case of iron deficiency anaemia RDW is increased with low MCV.

Vitamin B_{12} and folate deficiency anaemia RDW is increased.

In thalassaemia trait RDW is normal with low MCV. *Normal range:* 11.5–14.5%

Exercise

8

Absolute Eosinophil Count

Total eosinophil counts can be roughly calculated from the total and differential leukocyte counts, the staining properties make it possible to count them directly and more accurately in the counting chamber.

Reagents and Equipment

1. Eosinophil diluting fluid.
2. Neubauer counting chamber.
3. Anticoagulated blood.
4. WBC pipette.

Eosinophil Diluting Fluid (Dunger's fluid)

Eosin, aqueous 200 g/L	10 ml
Acetone	10 ml
Water	80 ml

Acid dye (eosin/phloxine) stains the granules, and water acts as a solvent; and also lysis the RBCs and other leukocytes. Eosinophils resist the lysis. Acetone prevents lytic action of water on eosinophil.

Procedure

Draw blood up to '1' mark of WBC pipette and eosinophil diluting fluid up to '11' mark (dilution factor 1:10) and wait for a few minutes. Discard the first few drops and charge the chamber. Allow some time for the cells to settle down and count the corner 4 large squares.

Calculation:

$$\frac{n \times 10}{0.1 \times 1 \times 4} = n \times 25 \text{ cells/cmm}$$

If the corner four large squares of both the chambers (totally 8 large squares) are counted, the accuracy of count is better and for calculation the formula $n \times 12.5$ cells/cmm is applied.

Normal range: 40–440 cells/cmm

Exercise

9

Differential Leukocyte Count

Differential leukocyte count (DLC) is the percent distribution of various WBCs in the peripheral blood so as to establish the relative frequency of different types of WBCs. But a limited number of cells are usually counted (100) in the peripheral smear and recorded in percentage. Counting of 200–500 leukocytes yields higher accuracy. This is carried out on Wright or Leishman stained smears.

In case of the smears prepared on glass slides, it is generally assumed that the distribution of leukocytes is random. The smears should not be too thin and the tail should be smooth. If the smear is too thin and rough edged spreader is used, the leukocytes accumulate at the edges and the tail. Even otherwise in a moderately thick smear, the polymorphs and the monocytes predominate at the margins and the tail. The lymphocytes would be in the middle region. This difference is mainly based on the stickiness, specific gravity and size of different WBCs. On unsatisfactory smears, DLC is not done. Various systems of performing DLC are adopted.

The film must be inspected from head to tail; 100 cells are counted in a longitudinal strip. If less than 100 cells are counted in one strip, then examine one or more additional strips. Each longitudinal strip represents the blood drawn from a small part of blood. If all the cells in such strips are counted, the DLC would approximate closely to the true DLC (Fig. 1.19a).

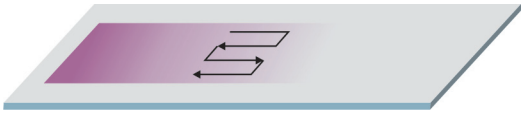


Fig. 1.19a: Method of inspection for doing DLC, peripheral smear

In the head part, sometimes it is difficult to identify the cells. The cells can be counted in a well spread area (body part of the smear) where the cells are clearly identifiable. The lateral edges are avoided.

Normal range

	Adults	*Children*
Neutrophils	60–70%	20–30%
Lymphocytes	20–40%	60–70%
Monocytes	02–08%	02–08%
Eosinophils	01–08%	01–08%
Basophils	00–01%	00–01%

Variations in Differential Count

Among children soon after birth for 3 days, polymorphs predominate and then they fall, whereas the lymphocytes predominate up to 5–7 years of age. However in tropical countries eosinophilia and monocytosis are common due to endemic parasites and protozoal diseases.

Causes for Variation in Leukocyte Counts

Neutrophilia (Fig. 1.19b)

The causes of neutrophilia

- Acute infections with cocci

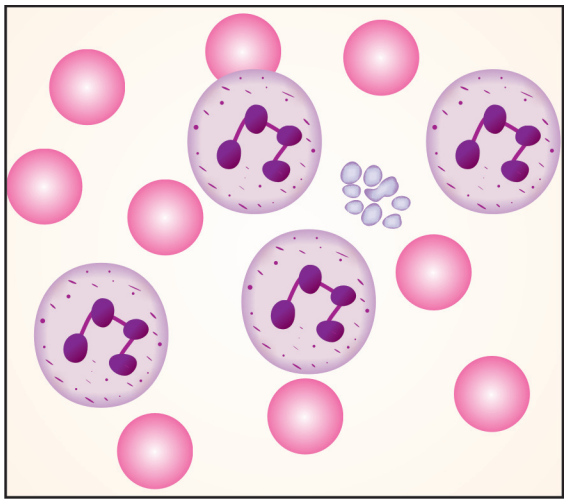


Fig. 1.19b: Neutrophilic leukocytosis

- Tissue injury—infarctions, burns, surgery and necrosis inducing processes
- Haemorrhage
- Neoplasms
- Stress states and hyperactivity conditions like convulsions, tachycardia, labour, severe colic, delirium-tremens
- Inflammatory disorders—collagen disorders, gout and rheumatic fever
- Metabolic disorders—diabetic ketoacidosis
- Corticosteroid administration
- Miscellaneous causes.

Lymphocytosis

Absolute lymphocyte count of about 4×10^9 cells/L is called lymphocytosis.

Relative lymphocytosis: Lymphocyte count is normal but when neutrophil count is reduced, there appears a relative increase in lymphocytes.

Causes of lymphocytosis

Viral—pertussis, mumps, measles, influenza and infectious mononucleosis. Atypical lymphocytosis is observed in EB virus infection, CMV infection and infective hepatitis. In atypical lymphocytosis, enlarged pleomorphic lymphocytes are observed. Turk cell is a reactive lymphocyte with basophilic cytoplasm and it resembles plasma cell.

Chronic bacterial infections like tuberculosis, syphilis, typhoid and such other chronic infections.

Eosinophilia (Fig. 1.20)

The causes of eosinophilia

- Allergy to extrinsic agents such as vegetables, animal products, parasites, drugs and blood products
- Neoplasms-lymphoproliferative malignancies (Hodgkin's disease), carcinomas
- Certain vasculitis and collagen disorders—polyarteritis nodosa
- Dermatological conditions—pemphigus and dermatitis herpetiformis
- Löeffler's syndrome
- Familial
- Post-splenectomy
- Miscellaneous.

Monocytosis

When the absolute monocyte count exceeds the limit of 0.8×10^9 cells/L, then that condition is called monocytosis.

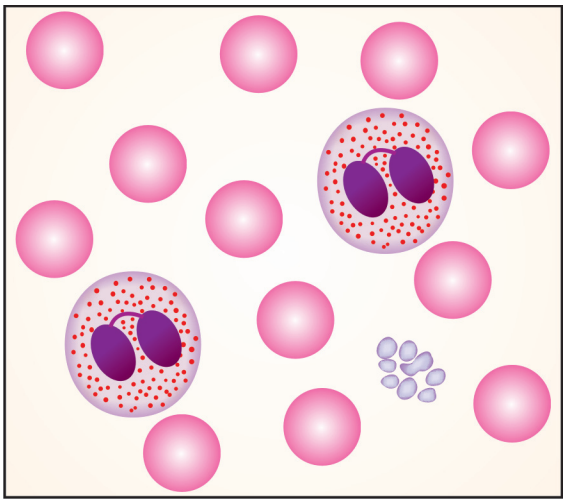


Fig. 1.20: Eosinophilia

Causes of monocytosis

- Infections like tuberculosis, malaria, bacterial endocarditis, typhoid, kala-azar, parasitic and protozoal diseases
- Ulcerative colitis
- Sarcoidosis
- Certain cases of acute myeloid leukemia
- Chronic myeloid leukemia
- Myelodysplastic syndrome.

Basophilia

Basophils are increased in chronic myeloid leukemia.

Lymphopenia

Absolute count below the limit of 1.5×10^9 cells/L is called lymphopenia.

Causes of lymphopenia

- Pancytopenia
- Advanced Hodgkin's disease
- Prodromal phase of viral infections due to depletion of helper T cells, e.g. AIDS
- Corticosteroid therapy.

Neutropenia

The causes of neutropenia

- Conditions that replace normal haemopoietic cells like acute leukemia, myelofibrosis, lymphoma, multiple myeloma, myelodysplastic syndrome
- Infections—typhoid, viral infections, sepsis
- Megaloblastic anaemia
- Aplastic anaemia
- Iron deficiency anaemia
- Drugs and radiation—marrow depression
- Chronic idiopathic neutropenia
- Hypersplenism
- Cytotoxic therapy
- Cyclic neutropenia.

Exercise

10

Packed Cell Volume (Haematocrit)

The volume of erythrocytes expressed as a percentage to the volume blood is called haematocrit (Fig. 1.21a).

Blood used: EDTA, double oxalate or heparinised blood.

Equipment

1. Wintrobe's haematocrit tube (110 mm long, internal bore—3 mm)
2. Pasteur's pipette
3. Centrifuging machine
4. Anticoagulated blood

Procedure

Mix the blood adequately and label the Wintrobe's tube (Fig. 1.21b). Fill the Wintrobe's haematocrit tube using Pasteur's pipette (Fig. 1.21c). As filling proceeds, the tip of the pipette is raised and filled up till 100 mark. Care is to be taken to see that there are no air bubbles present in the blood column. After the

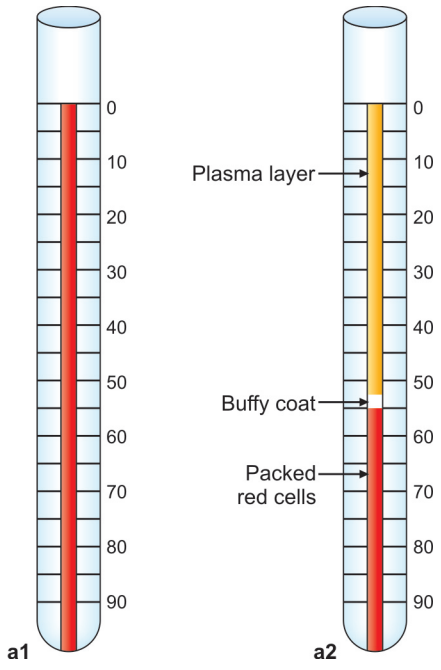


Fig. 1.21a: Schematic diagram of PCV (a1: Before centrifugation, a2: After centrifugation)

Fig. 1.21b Wintrobe's tube with stand

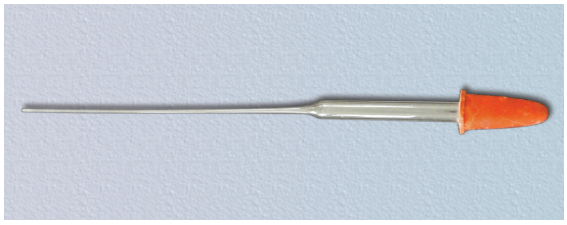


Fig. 1.21c: Pasteur's pipette for filling the Wintrobe's tube

blood is filled, it is preferred to cap the tube to avoid evaporation. Then the tube is placed in the centrifuging machine and centrifuged at 3000 RPM for 30 minutes. Reading is taken without disturbing the Wintrobe's tube. Read the red cell column and express in percentage or calculate by using the following formula.

Haematocrit percentage = 100 × L1/L2

L1 = Height of the red cell column in the tube

L2 = Height of whole blood (red cells + buffy coat + plasma layer)

While taking L1 reading, buffy coat layer is not included or read the red cell column and express in percentage.

Microhaematocrit Method

For this method a capillary haematocrit tube about 7.5 cm long with bore of 1 mm is used. Blood is filled by capillary action leaving 1.5 cm unfilled. The empty end is sealed by heating or filling soft wax/clay. It is centrifuged at 5000 to 12000 g/minute for 10 minutes for the former and 5 minutes for the latter. Special centrifuging machines are available for this method. The length of whole blood and length of RBC column are noted.

Normal values

Males	47 ± 7 (40–54%)
Females	42 ± 5 (37–47%)

PCV variations

- Decreased in anaemias and pregnancy.
- Increased in polycythemia, shock, dehydration, emphysema and congenital heart disease.

Errors in PCV could be due to

- Inadequate mixing of blood
- Improper reading of the levels of RBC column and plasma column
- Irregularity in the diameter of the bore of the tube
- Excess EDTA causes shrinkage of RBCs and PCV decreases
- PCV increases with tourniquet tied for longer duration while drawing blood.

PCV is simple screening test for anaemia. In conjunction with Hb% and RBC count, PCV helps in calculation of blood cell indices. Look for the RBC column, buffy coat and plasma layer in PCV carried out in Wintrobe's tube. Buffy coat is usually 0.5 to 1 mm; 0.1 mm denotes 1000 WBC cells/cmm.

Plasma layer

Reddish	Suggests haemolysis
Yellow	Suggests jaundice
Milky white	Suggests hypercholesterolemia
Cloudy	Indicates increased viscosity of plasma proteins as in multiple myeloma

Exercise

11

Erythrocyte Sedimentation Rate (ESR)

It is described as the rate of fall of column of the erythrocytes in a given period of time when blood is held in a vertical tube.

Westergren's Method (Fig. 1.22a)

Westergren's pipette with stand (Fig. 1.22b): Westergren's pipette is 30 cm long; its inside bore diameter is 2.5 mm; both ends are open; and markings are from 0 to 200 above downwards (graduated in the lower 20 cm). The pipette must be clean, dry and free from dust. It should be thoroughly washed with tap water, rinsed with acetone allowed to dry and it can be reused. Teat or mechanical devices for suction should be used. Mouth suction should be avoided. EDTA blood/citrated blood is used. As the length of the column is more, dilution is not much affected with the citrated blood.

Procedure

Pipette out the blood up to '0' mark. Allow it to stand in a vertical position in Westergren's

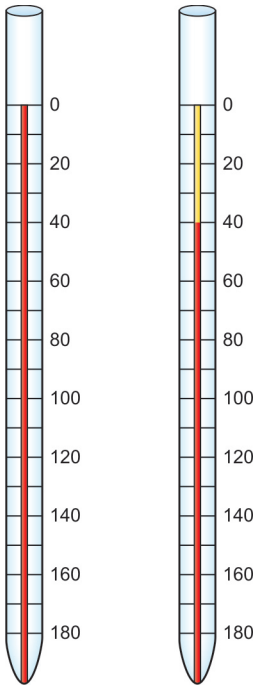


Fig. 1.22a: Schematic diagram of ESR by Westergren's method

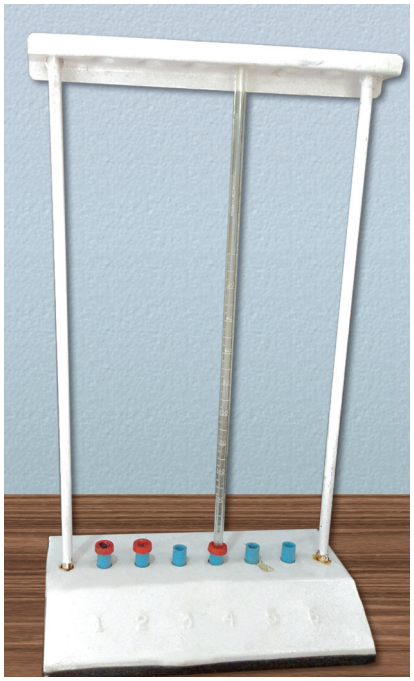


Fig. 1.22b: Westergren's pipette with stand

stand; read the upper level of the red cells exactly at the end of one hour. The reading is expressed as … mm at the end of the first hour.

Normal range

Males	5 to 15 mm/1st hour
Females	5 to 20 mm/1st hour

Wintrobe's Method

- *Blood used:* EDTA blood
- Wintrobe's tube with stand and Pasteur's pipette.

Wintrobe's tube: Markings are 0 to 100 above downwards (for ESR) and below upwards (for PCV)

Procedure

Fill the blood up to '0' mark with Pasteur's pipette. Allow it to stand vertically for one hour in Wintrobe's stand. At the end of one hour note the upper level of red cells.

Normal values

Males	0–10 mm/1st hour
Females	0–20 mm/1st hour

Micro Method

A plastic disposable tube of 230 mm long with 1 mm bore diameter is used. About 0.2 ml blood is needed. This method is useful in paediatric patients; procedure and reading taken are similar to the other methods.

Landau Method (Micro-sedimentation method)

Used in infants when blood is insufficient for the above methods. Capillary blood can be used.

Equipment

Landau pipette with stand—looks like RBC pipette with markings 0–50 mm.

5.0 g/dl sodium citrate is used as an anticoagulant.

Procedure

Draw sodium citrate up to the first mark on the stem and then blood up to the second mark. Wipe off excess blood on the tip of the pipette. Draw both the solutions in the bulb. Set the upper level of the mixture to '0' mark at the top. Detach the suction device, and then place the pipette in a vertical position on the stand. Note the reading at the end of the 1st hour.

Normal range

Male	0–5 mm/1st hour
Female	0–8 mm/1st hour

Stages in ESR

There are three stages in ESR.

- *Stage 1:* Stage of aggregation: During the first 10 minutes the red cells form rouleaux. The factors which influence rouleaux, greatly influence the ESR.
- *Stage 2:* Stage of sedimentation: In next 40 minutes the aggregated cells fall.
- *Stage 3:* Stage of packing occurs in last 10 minutes.

Factors Influencing ESR

1. Normally for ESR, room temperature of 18–25°C is preferred. With increase in temperature ESR also increases.
2. With the lapse of time and in stored blood, the ESR is reduced. Hence, it is preferable to do ESR within 4 hours of collection of blood.
3. Place the ESR pipette vertically; and free from vibrations and sunlight.
4. Length of tube: Sedimentation is better with long tubes.
5. Rouleaux formation is facilitated by globulins fibrinogen and acute phase proteins-haptoglobin, ceruloplasmin, α_1-antitrypsin, c-reactive protein, etc.
6. It is retarded by albumin.
7. Cholesterol to some degree increases ESR.
8. ESR is more in females than males because of the higher levels of fibrinogen.
9. ESR increases during pregnancy because of the increase in red cell aggregation.

10. ESR is influenced by stage of menstrual cycle and drugs like steroids and contraceptives.
11. With high blood cell counts, ESR is low and low blood cell counts increases the ESR by accelerating the rate of fall.
12. ESR is low in cases of polycythemia, hypofibrinogenemia, CCF, abnormalities of red cells such as poikilocytosis, spherocytosis and sickle cell anaemia. Microcytes resist rouleaux formation with the reduction in ESR.
13. ESR is low in infants.

Note

- Citrated blood (1 : 4 ratio) can be used for Westergren's method.
- Because of biohazard in cases of HIV, hepatitis B, etc. instead of open ended tubes, closed systems are highly recommended for ESR.

Exercise

12

Blood Groups Related Exercises

BLOOD GROUPS

There are several blood groups; they are ABO, MNSs, P, Rh, Lutheran, Kell, Lewis, Kidd, Duffy, Diego, Yt, Xg, Ii, Dombrock, etc. Amongst these, ABO and Rh systems are important.

ABO BLOOD GROUPS (Fig. 1.23)

The red cell surface has antigens. The antigenic characters of red cells are inherited. The antigen detection of blood groups is based upon haemagglutination reactions. This is a serological reaction of red cells with the corresponding antibody, as determined in the laboratory. There are naturally occurring ABO group antibodies of IgM type in the serum of the patients. The serum contains the antibody for that antigen missing on the cell surface.

Some information about ABO system is given below.

Blood group	*General population*	*Antigens*	*Antibodies*
AB	3%	A and B	Nil
A	42%	A	Anti-B
B	8%	B	Anti-A
O	47%	Nil	Anti-A, Anti-B

Note: Racial variations in the frequency of these groups are noticeable.

Purpose of ABO blood grouping

i. Blood transfusion
ii. Medicolegal purposes, i.e. in cases of disputed paternity.

Procedure

Red cell suspension with 0.9% NaCl is prepared. Take a slide with three concavities

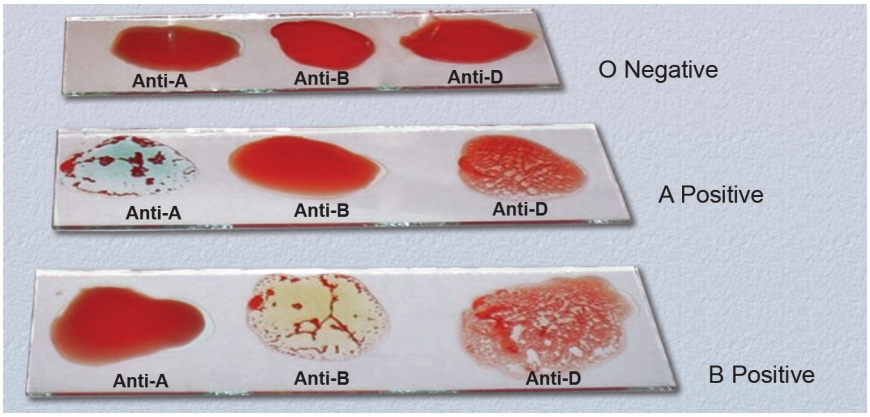


Fig. 1.23: Blood groups by slide method

or two concavities. Label the concavities as anti-A and anti-B. In a slide with three concavities, the central one is used for control.

One drop of anti-sera A (blue coloured) is put in the concavity labelled as anti-A and one drop of anti-sera B (yellow coloured) in concavity labelled as anti-B. Add one drop of blood diluted with normal saline to all the concavities. The central concavity containing only saline diluted blood acts as control. Take a glass rod; mix well, each time using different ends. Care is taken not to contaminate one another. Observe for agglutination after 5 to 10 minutes.

Agglutination	*Blood group*
Present in both anti-A, anti-B concavities	'AB'
Absent in both concavities	'O'
Present in only anti-A concavity	'A'
Present in only anti-B concavity	'B'

Note: When doubtful agglutination is present, the agglutination may be checked under microscope.

The antibodies in ABO system are naturally occurring complete antibodies and they can be easily detected by saline agglutination tests.

Blood Group Antigens

The antigenic determinants or epitopes are small portions of molecules recognised by antibodies.

ABO antigens are carbohydrate in nature. They are oligosaccharide chains anchored to glycoproteins or glycolipids of the RBC membrane.

They are highly immunogenic.

A and B antigens differ in only the terminal sugar.

There is terminal sugar N-acetyl galactosamine in A group.

And terminal sugar galactose in B group.

There is no A or B antigen in O blood group.

Antibodies

In ABO system there are natural antibodies.

They are present in the serum/plasma.

They are IgM type and have high molecular weight and hence cannot pass through the placenta.

These antibodies react well at 4°C, room temperature or at 37°C.

Sometimes IgG antibodies may be produced in O blood group patients which can cause hemolytic transfusion reactions and hemolytic disease of newborn.

UNIVERSAL DONORS AND RECIPIENTS

The earlier concept of 'O' blood group as universal donor and AB blood group as universal recipient does not hold good.

'O' blood group person have no antigens on the red cells but have anti-A and anti-B antibodies in the serum.

When given to recipient, these antibodies can destroy some of the recepient's red cells.

Hence, 'O' blood, better not to be given to A, B or AB persons. However, washed red cells can be given.

Earlier notion of AB person as a universal recipient, as they have A and B antigen on the red cells does not hold good. A or B blood groups will have antibodies against AB blood group antigens and can destroy some red cells of the AB recipient. Hence, A or B blood groups better not to be transfused to AB blood group patient. However, AB plasma can be given to A, B or O persons as it does not have any antibodies.

Hence, washed O red cell packs and AB plasma are universal donors.

ABO SUBGROUPS

Subgroups of A: A_1 and A_2

These two phenotypes are best differentiated using lectin that is extracted from the seeds of *Dolichos biflorus* which reacts only with A_1 cells.

A_1 accounts for 80% of blood group A.

A_2 accounts for 20% of blood group A.

A_2 reacts weakly and misdiagnosed as 'O' blood group.

A_2 gene has two nucleotides different from A_1 gene which results in diminished enzymatic activity and subsequently weakened antigen expression.

If A_2 is misdiagnosed as 'O' blood group, there is no harm; however if A_2 is misdiagnosed as 'O' and if this blood is given to 'O' recipient, the anti-A and anti-B antibodies of the recipient might cause the early destruction of transfused blood.

A_2 and A_2B individuals can produce anti-A_1 antibodies.

Approximately 4% of A_2 individuals and up to 25% of the A_2B individuals can have Anti-A_1 antibodies in their serum.

The number of other subgroups of A has been described. This appears to result from inheritance of rare alleles of ABO locus and include A_{int}, A_3, A_x, A_m, A_{end}, A_{el}, A_{buntu} and A_{finn}. Except for A_{int} and A_3, many of these subgroups are weakly reactive or non-reactive with anti-A antibodies.

Subgroups of B

As described for A blood group, subgroups of B are also reported. Reactions of these red cells with anti-B are weak and variable.

BOMBAY BLOOD GROUP

This phenotype arises when two hh genes are inherited at the Hh locus. Such individuals are unable to convert type II paragloboside to H antigen. Hence, they are unable to make A or B antigens. These individuals produce anti-H, anti-A and anti-B as naturally occurring antibodies. On initial testing, Bombay blood group red cells appear to be of group 'O' but when this blood is transfused to 'O' blood group patients, these patients produce haemolytic reactions. This blood group occurs 1 in 13000 population all over.

LABORATORY TESTS DONE ON THE UNIT OF BLOOD DONATED

Following are the tests:

1. Haemoglobin estimation.
2. Blood grouping and cross matching.
3. Screening for unwanted antibodies.
4. Screening for transfusion transmissible infections: Indian Govt (Food and Drug Control Act) recommends following 5 tests to be mandatory. These are mentioned below.
   - HIV 1 and 2
   - Hepatitis B
   - Hepatitis C
   - Syphilis
   - Malaria.

   Tests must be performed at each donation regardless of number of earlier donations.

TRANSFUSION REACTIONS

Transfusion reaction is defined as any unfavorable event that occurs during or after a transfusion of blood and its components.

The transfusion reactions can be classified as

Acute transfusion reactions—immunological

- Febrile non-hemolytic transfusion reactions (FNHTRs)
- Allergic reactions
- Anaphylactic and anaphylactoid reactions
- Acute hemolytic transfusion reactions (AHTRs)
- Transfusion related lung injury (TRALI).

Acute transfusion reactions—non-immunological

- Bacterial contamination
- Transfusion-associated
- Circulatory overload (TACO)
- Physical and chemical
- Hemolysis
- Metabolic derangements.

Delayed transfusion reactions—immunological

- Transfusion-associated graft-versus-host disease (TA-GVHD)
- Post-transfusion purpura.

Delayed transfusion reactions-non-immunological

- Iron overload
- Transfusion-transmitted diseases.

RH SYSTEM (RH TYPING)

The Rh system is so named because the original antibody was raised by injecting red

cells of rhesus monkeys into rabbits and guinea pigs, also reacted with human cells. The Rh system is a gene complex which gives rise to various combinations of three alternative antigens C or c, D or d and E or e as originally suggested by Fisher. The Rh locus is on chromosome 1. Amongst these antigens, D antigen is the most immunogenic and it is convenient to classify the individual as Rh-D positive or Rh-D negative, depending on the presence of the Rh-D antigen. For Rh-D antigen detection, usually slide agglutination procedure is routinely done; whenever doubt arises tube technique is followed.

Slide Agglutination Method

One drop of anti-D and one drop of blood are mixed well, observe for agglutination after 2 minutes. This can be done along with ABO grouping as shown in Fig. 1.23.

Note: False negative result may be observed when room temperature is less and the test may need pre-warming of the slide.

Rh Confirmation by Tube Technique

This is done with controls; wash the cells with saline 3 times (5 drops of blood). Take 1 drop of cell suspension and 1 drop of anti-D; incubate at least for 30 minutes. Add anti-human globulin serum. Centrifuge for 1 minute and observe for agglutination.

WEAK D PHENOTYPE (DU PHENOTYPE)

Because of immunogenicity, the D antigen is the most clinically important antigen in the Rh blood group system. The donor and the recipient are tested for the presence or absence of the D antigen. The D positive recipients can receive D positive blood components and they can as well receive D negative blood components. On the other hand D negative recipients should be transfused with only D negative blood components. Although D typing on the vast majority of blood samples is straight forward, some variants of weak D typing may be encountered. These weak D typings are usually labelled as D negative on an immediate spin reading, but they are D positive when indirect antiglobulin test is conducted. This weak variant is described as Du phenotype (weak D). Reasons for this variant include a transposition effect, genetically transmissible Du and D categories.

When a C-producing Rh gene (without D) is in transposition, weakened expression of D antigen may be observed. These cells may fail to react with anti-D sera at immediate spin but they react strongly at antiglobulin phase of testing. This type of Du is also called high grade Du.

Some Du phenotypes arise from inheritance of specific Rh genes. This type Du is referred to as the low-grade Du. Among the blacks, a variant of R0 gene may produce lesser amounts of D antigen. Among the whites such diminished production is more frequently associated with variant R1 or variant R2 gene.

Among individuals with alloanti-D in the serum of D positive individuals, D Ag is proposed to be a mosaic, composed of genetically distinct pieces. A majority of D positive individuals have inherited Rh genes that produce all pieces of the mosaic; however some may inherit most of the pieces but not all the pieces of the antigen. Such individuals are at a risk during pregnancy and transfusion, to produce anti-D to the portion of D antigen, they lack on their red cells. These are grouped as D categories. Some of these are D positive on immediate spin, however others appear to be D-negative on immediate spin and demonstrate positive D with antiglobulin phase of testing. So most D category individuals are not apparent until they present with alloanti-D in their serum.

Hence, Du testing for donor cells is necessary to avoid immune response if transfused to D-negative recipient.

CROSS MATCHING

Purpose

This is done to ensure absence of incompatibility between the blood to be transfused and the blood of recipient.

Major cross matching is important, in which the serum of the recipient and the cells of the donor are mixed. The purpose of the major cross match is that the recipient's serum should not contain iso-antibodies to the donor's red cells. Minor cross matching is meant to detect iso-antibodies in the serum of the donor because they are capable of reacting with the recipient's red cells. This test is not mandatory.

Procedure of Major Cross Match

Prepare 2% red cell suspension of donor cells in saline. Add 2 drops of recipient's serum and 2 drops of red cell suspension, centrifuge at 1500 RPM for one minute and check for agglutination both macroscopically and microscopically. If positive the test detects IgM antibodies.

If there is no agglutination, then incubate in waterbath at 37°C for 15 minutes. Wash 3 times with saline. Thereafter follow the procedure of Coombs' test. Check for agglutination; if positive, it denotes IgG antibodies.

Procedure of Minor Cross Match

Similar procedure as above is followed using red cells of the recipient and the donor's serum.

COOMBS' TEST

Purpose: This test detects incomplete antibodies (IgG).

Requirements: Small glass test tubes (10 × 75 mm), pipettes, normal saline, centrifuging machine, Coombs' serum and the blood to be investigated into.

Coombs' serum (anti-human globulin serum): This is obtained by immunizing rabbits with human serum. Broad-spectrum antisera contains anti IgG and anti-complement components. Specific antisera against heavy chains of IgG, IgM and IgA can be prepared.

Direct Coombs' Test

Wash the test red cells 3–4 times with minimum of 3 ml of saline per wash and prepare 10–20% of red cell suspension in saline. About 2 drops of red cell suspension and 2 drops of Coombs' serum are mixed. Wait for 5 minutes. Centrifuge for 1 min/1500 RPM. Check for agglutination with naked eye or under microscope.

Test can be conducted with fourfold dilution of Coombs' serum (1:4, 1:16, 1:64, 1:256, 1:1024, and 1:4096).

Coombs' test with broad-spectrum antisera is non-specific. It would agglutinate a wide range of proteins, drugs and corresponding antidrug antibodies.

Indications

- Haemolytic disease of the newborn
- Auto-immune haemolytic anaemia
- Haemolytic transfusion reaction (incompatible blood transfusion).

Indirect Coombs' Test

Prepare red cell suspension of a known antigenicity ('O' cells). In a test tube place 2 drops of serum to be tested. To this add 2 drops of 10–20% red cells suspension ('O' blood group cells). Incubate for 30 minutes to 2 hours. If no agglutination, then wash for 3 times. Thereafter follow the steps of direct Coombs' test.

Indications

1. Detection of IgG antibodies to Rh factor (pregnant patients).
2. Detection of auto-antibodies in the serum of patients with auto-immune haemolytic anaemia.

Note: These tests should be conducted with controls.

Sources of errors

1. Red cells need to be washed adequately before adding anti-human globulin serum. Otherwise neutralization of anti-human globulin serum may occur.
2. Adequate incubation period is necessary.

Exercise

13

Normal Blood Picture

Reporting of Normal Blood Picture (Fig. 1.24)

RBCs: Normocytic and normochromic

WBCs: Normal in count and distribution.

Platelets: Adequate and seen in clumps

Note:

1. Normal blood picture does not show variation in size (anisocytosis) and shape (poikilocytosis) of the RBCs.
2. To comment on the RBC size, bring a small lymphocyte in the field and compare with its size. The RBC is called normocytic, if its size is almost same size as that of a small lymphocyte.
3. To label the RBC as normochromic, pallor should be central 1/3. This central pallor is due to biconcave shape of the RBC.
4. Usually one WBC per oil immersion (100 X magnification) is seen when WBC count is within normal limits.

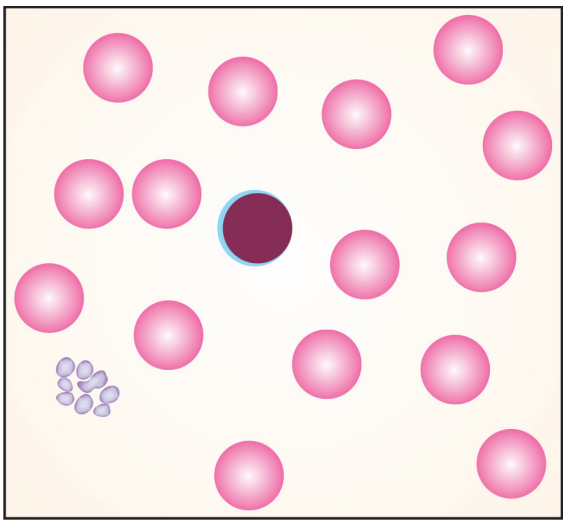


Fig. 1.24: Peripheral smear of normal blood picture (schematic)

5. Platelets are always seen in clumps in peripheral smear and one clump per oil immersion may be seen when platelet count is within normal limits.

Exercise

14

Nutritional Anaemias

IRON DEFICIENCY ANAEMIA

Iron deficiency anaemia occurs due to either reduced intake, decreased intestinal absorption, increased utilization or chronic blood loss. As a result, there is reduction in concentration of Hb in circulating blood below normal for that particular age and sex.

Causes

Major etiological factors for iron deficiency anaemia are:

Females in reproductive life

- Pregnancy—number and frequency
- Miscarriages
- Lactation
- Pathological blood loss
  - Deficient diet
  - Inadequate iron intake.

Adult males and postmenopausal females

- Pathological blood loss—causes are mentioned below.

Infants and children

- Deficient diet/inadequate iron intake (mentioned below)
- Diminished iron stores at birth.

Inadequate iron intake: This is the major cause of iron deficiency anaemia in infants and children. In adults it may occur due to:

- Poor economic status
- Iron content may be lower with vegetarian diet
- Dietary fads or dislikes.

Pathological blood loss

- Menorrhagia
- GI bleeding
  - Peptic ulcer
  - Carcinoma stomach
  - Carcinoma colon
  - Chronic aspirin ingestion/NSAID use
  - Oesophagitis
  - Oesophageal varices
  - Haemorrhoids
  - Hookworm infestation
  - Hiatus hernia
  - Angiodysplasia
  - Diverticulosis
  - Meckel's diverticula
  - Colitis or inflammatory bowel disease
- Bleeding disorder
- Pulmonary lesions with bleeding
- Haemoglobinuria—haemosiderinuria (chronic intravascular haemolysis)
- Haemodialysis
- Haematuria (chronic)
- Frequent blood donation each time 200–250 mg iron/unit-blood is lost.

The reasons for decreased absorption of iron are

- Gastric surgery
- Achlorhydria
- Sprue/coeliac disease
- Pica (non-nutritive substances like clay, chalk, sand, ice, etc.).

Clinical features: Clinical features most commonly occur with long-standing iron deficiency states.

Following are the clinical features

- Pallor, fatigue, weakness, dyspnoea
- Anxiety, irritability, angina, sleepiness, palpitations
- Changes in the tongue-like atrophy of papillae resulting in pale bald tongue
- Changes in the nails—longitudinal ridging, flattening and koilonychia (spoon-shaped nails) or nails that are weak or brittle
- Poor appetite
- Unusual obsessive food cravings, known as pica
- Plummer-Vinson syndrome (Paterson-Brown Kelly syndrome): Dysphagia due to formation of oesophageal webs, iron deficiency anaemia, glossitis, cheilitis and splenomegaly; most commonly seen in postmenopausal females
- Tayanc-Prasad syndrome (growth retardation, hypogonadism, hepatosplenomegaly, zinc and iron deficiency, geophagia).

Approach to a Patient with Iron Deficiency Anaemia

History

Females in reproductive period: Menorrhagia, pregnancies, number and frequency, miscarriages, iron deficient diet, GI blood loss, hematuria, epistaxis, haemoptysis, GI surgery, aspirin ingestion.

Males and postmenopausal females: Iron deficient diet, haematemesis, malaena or pre rectal bleeding (GI blood loss due to hemorrhoids, oesophageal varices, bleeding due to GI malignancies), haematuria, epistaxis, haemoptysis, GI surgery, aspirin ingestion.

Infants and children: Dietary history regarding supplementary feeding, prematurity, multiple births, iron deficiency in mother, GI disturbances, blood loss of any cause.

Physical and systemic examination: Examination any mass, rectal examination, pelvic examination in females, telangiectasias of face and mouth.

Relevant investigations commonly required

Examination of faeces for occult blood and hookworm.

Urine microscopy for haematuria

GI endoscopy or barium swallow study: Peptic ulcer, hiatus hernia, Ca stomach, oesophageal varices, Meckel's diverticulum.

Barium swallow studies in oesophageal varices in a cirrhotic patient show multiple serpiginous filling defects of lower one-third of the oesophagus.

Colonoscopy: Carcinoma colon, caecum, ulcerative colitis, diverticula, angiodysplasia

Sigmoidoscopy: Carcinoma rectum, ulcerative colitis.

Relevant investigations occasionally required

Chest X-ray and bronchoscopy (haemoptysis)

Cystoscopy (haematuria)

Liver function tests (cirrhosis).

Blood Picture, Bone Marrow and Biochemical Findings in Iron Deficiency Anaemia

1. *Complete blood count*
   - Low haemoglobin
   - Low haematocrit
   - Reduced RBC count.
2. *RBC indices*
   - Low MCV
   - Low MCH
   - Low or normal MCHC
   - Increased RDW.
3. *Peripheral smear* (Fig. 1.25)
   - RBCs: RBCs show anisocytosis and poikilocytosis.
   - Majority of the RBCs are microcytic hypochromic, ring/pessary type.
   - Pencil-shaped, target cells, tear drop cells polychromatic cells are present.
   - *WBCs:* Count and distribution normal.
   - *Platelets:* Count and morphology normal.

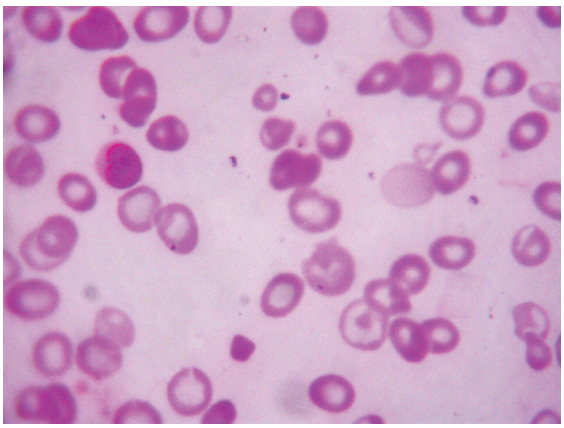


Fig. 1.25: Peripheral smear of microcytic RBCs in microcytic hypochromic anaemia

4. *Bone marrow examination:* Depleted iron stores (Perl's stain)
   - Erythroid hyperplasia
   - Micronormoblastic
   - Cytoplasm lags maturation
   - Granulopoiesis—normal
   - Megakaryopoiesis—normal.
5. *Iron studies*
   - Serum iron: ↓
   - Serum ferritin ↓ in general, values less than 10 µg/L are indicative of iron deficiency
   - *TIBC:* ↑, TIBC is 1/3 saturated under normal conditions
   - Plasma transferrin
   - *Transferrin saturation:* (Normal 6–33%), <5% definitely indicates iron deficiency
   - *Transferrin receptor:* Free erythrocyte protoporphyrin ↑.

Normal values

Serum iron: Male 27–138 µg/dl, female 33–102 µg/dl

Serum ferritin: Male 29–248 µg/L, female 10–150 µg/L

TIBC: Male 174–351 µg/dl, female 194–372 µg/dl

Plasma transferrin: Male 194–348 µg/dl, female 181–416 µg/dl

Free erythrocyte protoporphyrin: 17–27 µg/dl.

Differential diagnosis for microcytic anaemias (Fig. 1.26)

- Iron deficiency anaemia
- Thalassaemia, HbC, HbE, etc.
- Sideroblastic anaemia
- Lead poisoning
- Anaemia of chronic diseases (sometimes).

Grading of Iron Stores in Bone Marrow[1]

0	No iron granules seen
1+	Small granules in reticulum cells seen only with oil immersion
2+	Few small granules in reticulum cells seen only with low power
3+	Numerous small granules in all cells
4+	Large granules in small clumps
5+	Dense large clumps of granules
6+	Large deposits obscuring marrow picture.

MEGALOBLASTIC ANAEMIAS

Megaloblastic anaemias are macrocytic anaemias characterised by distinctive cytological and functional abnormalities in peripheral blood and bone marrow cells due to impaired DNA synthesis, resulting in erythroid precursors that are enlarged and show failure of nuclear maturation (megaloblasts).

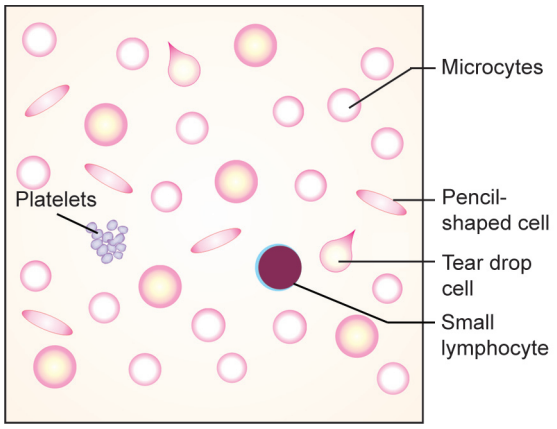


Fig. 1.26: Schematic diagram of microcytic hypochromic anaemia

Etiology: Megaloblastic anaemias result from conditions in which nucleic acid synthesis is abnormal as in:

- Vitamin B_{12} deficiency
- Folic acid deficiency.

Vitamin B_{12} is mainly obtained from foods of animal origin; kidney, heart and liver are richest sources. Lesser amounts are present in muscle meats, fish, eggs, cheese, and milk. Vegetarian diet has no B_{12}. The B_{12} is in the form of adenosylcobalamin and hydroxocobalmin and these are bound to proteins in the food. Folate is present in diet, largely attached to methyl group and is in inactive form. It is distributed in plant and animal tissues. The richest sources are liver, kidney, yeast and green leafy vegetables. Spinach and cabbage have good source of folates. Milk has low folate content (Table 1.1).

Absorption of vitamin B_{12}: When food passes through the stomach, vitamin B_{12} is released from the dietary proteins by the action of acid and proteolytic enzymes.

Vitamin B_{12} first combines with R protein released from the saliva. As this B_{12} and R complex proceeds to small intestine, the R protein is degraded by pancreatic enzymes and B_{12} is released. The B_{12} rapidly combines with the intrinsic factor (IF) secreted by parietal cells of fundus and body of stomach. B_{12} and IF complex as it passes in the ileum which is site of absorption. The B_{12} and IF complex binds to the receptors on the surface of the brush border cells and B_{12} is taken up. B_{12} in the circulation will be bound to transport protein called transcobalamine II. Transcobalamin I acts as storage protein. B_{12} is required for: Conversion of homocysteine to methionine.

Absorption of folate: Folate is absorbed from the duodenum and upper jejunum and to a lesser extent from lower jejunum and ileum. The polyglutamate are cleaved to monoglutamate and undergo further reduction and methylation and circulates in the blood as methyl tetrahydrofolate.

Folate is stored in the liver in polyglutamate form. It is required for

- Methylation of homocysteine to methionine
- Synthesis of thymidine monophosphate from deoxyuridilate monophosphate in DNA synthesis.

Role of Vitamin B_{12} and Folic Acid (Fig. 1.27)

Causes for megaloblastic anaemia due to B_{12} deficiency and folate deficiency are given in Tables 1.2 and 1.3.

Clinical Features

These patients present with general features of anaemia.

Following are the other features

- Glossitis
- Peripheral neuropathy and subacute combined degeneration of spinal cord in B_{12} deficiency anaemia
- Dementia
- Folate deficiency may also cause diarrhoea and glossitis.

Table 1.1: Information about vitamin B_{12} and folic acid

	B_{12}	*Folic acid*
Availability in diet	Vegetarian: Poor Non-vegetarian—meat: Rich	Vegetarian: Rich Non-vegetarian—meat: Moderate
Effect on cooking	10–30% loss	60–90% loss
Daily requirement in adults	2–4 µg	200 µg
Daily intake in adults	5–30 µg	100–500 µg
Absorption site	Ileum	Duodenum and jejunum
Body stores	2–5 mg	5–20 mg

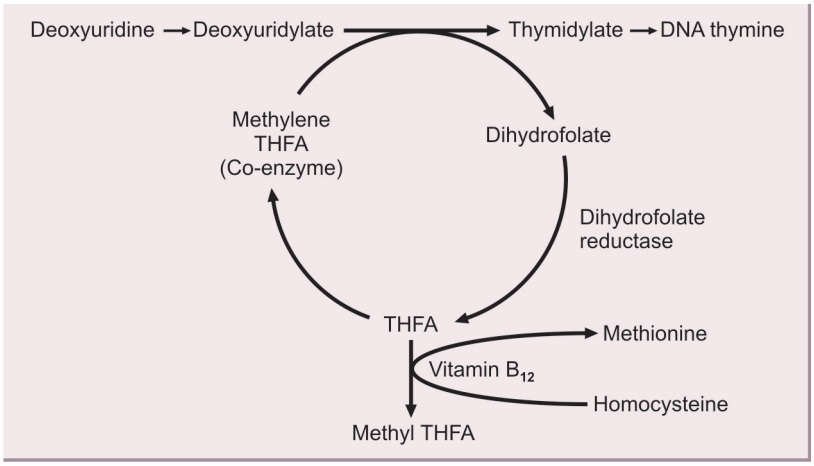


Fig. 1.27: Role of vitamin B_{12} and folate in DNA synthesis

Table 1.2: Causes of megaloblastic anaemia due to vitamin B_{12} deficiency

Mechanism	*Disorder*
Decreased intake	Nutritional deficiency
Impaired absorption	Gastric causes
	Pernicious anaemia
	Gastrectomy—total or partial
Intestinal causes	Lesions of small intestine
	Coeliac disease
	Tropical sprue
	Fish tapeworm infestation
	Bacterial overgrowth (blind loop syndrome)
	Surgical resection of Ileum

Table 1.3: Causes of megaloblastic anaemia due to folate deficiency

Mechanism	*Disorder*
Decreased intake	Nutritional deficiency
Impaired absorption	Lesions of small intestine
	Coeliac disease
	Tropical sprue
Increased demand	Pregnancy, puerperium, haemolytic anaemia, sideroblastic anaemia, MPDs, leukemias and lymphomas, carcinoma hyperthyroidism
Drugs	Anti-folate drugs (anti-epileptics), DHA reductase drugs (Methotrexate), alcohol

Pathology

Red cell changes

- Hb is moderately to markedly reduced, in the range of 5–10 g/dl, may go down as below as 2–3 g/dl
- PCV is reduced
- MCV >100 fl
- MCH is increased
- MCHC is normal
- Reticulocyte count is normal or slightly increased (2–3%).
- Erythropoiesis changes from normoblastic to megaloblastic.

Megaloblasts differ from normoblasts and show nuclear-cytoplasmic asynchrony.

- They are larger (increased cytoplasm) and
- Show delayed nuclear maturation
- But have normal cytoplasmic haemoglobinization.

Peripheral smear (Fig. 1.28)

RBCs: There is moderate to marked anisocytosis and poikilocytosis.

There is macrocytosis (large red cells with elevated MCV) and marked variation in size (anisocytosis) and shape (poikilocytosis)

- Oval forms (macro-ovalocytes) are prominent
- Evidence of dyserythropoiesis
  - Basophilic stippling
  - Cabot ring, Howell-Jolly bodies.

Megaloblastic anaemias are therefore macrocytic anaemias if morphologic classification is used.

Few nucleated RBCs with megaloblastic change may be seen.

Changes in white blood cells: Neutrophils show hypersegmented nuclei, with many cells showing more than 5 nuclear lobes.

Platelets: Normal or reduced.

Pancytopenia is seen in 10–20% cases of megaloblastic anaemias.

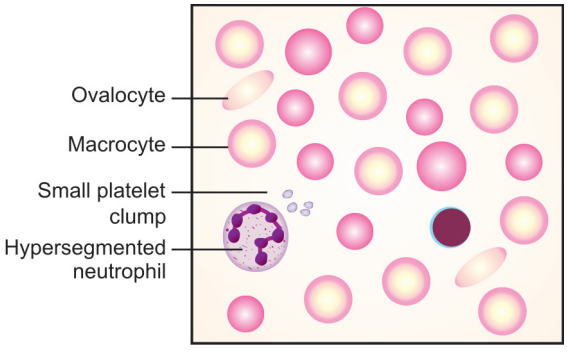


Fig. 1.28: Peripheral smear in megaloblastic anaemia to show macrocytes and hypersegmented neutrophil

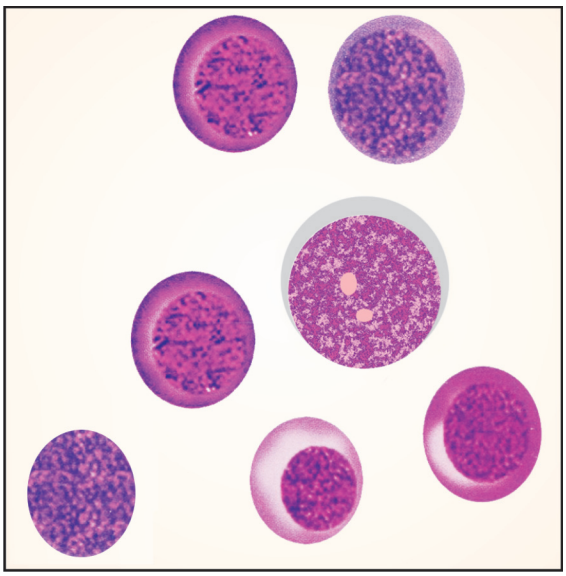


Fig. 1.29: Bone marrow in megaloblastic anaemia: Megaloblasts and other erythroid series cells with nuclei showing open chromatin

Bone Marrow Changes (Fig. 1.29)

Megaloblastic marrow

- These are large cells compared to normal nucleated erythroid precursors
- The nucleus has open-sieve like chromatin
- There is evidence of dyserythropoiesis
- Nuclear maturation lags behind the cytoplasmic maturation
- Late normoblasts have open chromatin
- Giant metamyelocytes are present
- Mitosis increased
- Marrow is hypercellular
- M:E ratio increased (1:1 or 2:1)
- Megakaryocytes may be reduced
- In pure megaloblastic anaemia iron stores may be increased.

Delayed maturation leads to accumulation of erythrocyte precursor cells. The bone marrow is hypercellular and contains large numbers of megaloblasts; as a result of intramedullary haemolysis or ineffective erythropoiesis, many megaloblasts undergo destruction in the bone marrow before maturation and this:

- Aggravates anaemia with
- Mild elevation of serum bilirubin and lactate dehydrogenase (LDH isoenzymes 1 and 2).

Megakaryocytic series are also affected.

Due to affection of all the series and ineffective erythropoiesis there may be pancytopenia, leukopenia or thrombocytopenia in these patients.

Megaloblastic anaemia should be suspected upon finding in the peripheral blood:

- Macrocytic anaemia with
- Hypersegmented neutrophils.

Biochemical and other Investigations

Serum B_{12} levels decreased in B_{12} deficiency anaemia.

Serum level and urinary excretion of methyl malonic acid are increased in B_{12} deficiency.

Homocysteine levels increased in both B_{12} and folic acid deficiency

Serum bilirubin increased in both B_{12} and folic acid deficiency.

Serum LDH is increased in both B_{12} and folic acid deficiency.

Serum ferritin levels are increased in both B_{12} and folic acid deficiency.

Normal values

Serum cobalamin levels—200–900 ng/L, <100 ng/L in megaloblastic anaemia due to vitamin B_{12} deficiency

Serum methyl malonic acid > 0.4 µmol/L

Serum folate levels up to 5.0 µg/L, < 3 µg/L in megaloblastic anaemia due to folate deficiency

Homocysteine levels—males 14–15 µmol/L, females—12–14 µmol/L

Red cell folate levels >160 µg/L.

Schilling Test in B_{12} Deficiency

1st step

Radioactive (RA) B_{12} (58 Co-B_{12}) is given orally.

Immediately 1,000 µg of non-RA B_{12} is given by IM to saturate B_{12} binding proteins.

Urine is collected for 24 hours

In normal health more than 10% of RA B_{12} is excreted in urine.

2nd step

If this is abnormal, the test is repeated with IF.

Interpretation: If the test turns normal with IF, the diagnosis of pernicious anaemia is made or IF deficiency may be because of gastrectomy.

If still abnormal it is because of Ileal pathology or blind loop syndrome.

Microbiological Assay

Two microorganisms *Euglena gracilis* and *Lactobacillus leichmani* are B_{12} dependent organisms and B_{12} in the serum is determined by comparing the growth of the organisms.

Deoxyuridine Suppression Test

Serum folate levels are decreased in folate deficiency anaemia.

Red cell folate levels—decreased

FIGLU an intermediate product in conversion of histidine to glutamate and is excreted in urine in folate deficiency.

Microbiological Assay

The folate activity can be assessed by methyl tetrahydrofolate; this compound is microbiologically active for *Lactobacillus casei* which is used for assay.

Diagnosis of Megaloblastic Anaemias

- Oval macrocytes in peripheral smear
- Hypersegmented neutrophils
- Megaloblastic hypercellular marrow
- Response to B_{12}/folate therapy.

Other Causes of Macrocytic Anaemia

- Alcoholism
- Hepatic causes
- Hypothyroidism
- Increased retic count—haemolysis
- Drugs.

Exercise

15

Haemolytic Anaemias and Tests Related to Haemolytic Anaemias

General Aspects

Haemolytic anaemia results from premature destruction of erythrocytes. The normal red cell lifespan is 120 days. In haemolytic anaemia the lifespan of RBCs is shortened by varying degrees and in many cases they survive only for a few days.

Patient may not always be anaemic because of bone marrow compensation.

Anaemia in haemolytic anaemia develops due to:

- Reduced lifespan
- Aplastic crises
- Haemolytic crises.

Clinical Features

- Pallor
- Intermittent jaundice
- Splenomegaly
- Gallstones—in chronic forms
- Crisis—aplastic, haemolytic
- Ankle ulcers.

Classification of Haemolytic Anaemia (HA)

HA due to intrinsic (intracorpuscular) abnormalities

Congenital

- Membrane abnormalities
  - Membrane skeleton proteins: Spherocytosis, elliptocytosis
  - Membrane lipids: Abetalipoproteinemia
- Disorders of haemoglobin synthesis
  - Deficient globin synthesis: Thalassaemia syndromes
  - Structurally abnormal globin synthesis (haemoglobinopathies): Sickle cell anaemia, unstable haemoglobins
  - Double heterozygous disorders: Sickle cell beta thalassaemia
- Enzyme deficiencies
  - Glycolytic enzymes: Pyruvate kinase, hexokinase, enzymes of hexose monophosphate shunt: Glucose-6-phosphate dehydrogenase, glutathione synthetase.

Acquired

Membrane defect: Paroxysmal nocturnal haemoglobinuria

HA due to extracorpuscular abnormalities

Acquired

- Immune mechanisms
  - Antibody mediated—warm antibodies/cold antibodies
  - Transfusion reactions: Incompatible blood transfusion
  - Erythroblastosis fetalis (Rh disease of the newborn)
  - Autoantibodies: Idiopathic (primary), drug-associated, systemic lupus erythematosus
- Non-immune mechanisms
- Mechanical trauma to red cells

Microangiopathic haemolytic anaemias: Thrombotic thrombocytopenic purpura, disseminated intravascular coagulation

- Miscellaneous causes
  - Infections: Malaria
  - Burns
  - Lead poisoning.

While investigating a case of haemolytic anaemia following questions needs to be answered

1. Is the anaemia of haemolytic nature?
2. If haemolytic anaemia is present, what is the site of destruction?
   Intravascular or extravascular?
3. What is the aetiology?

The haemolytic nature is determined by

1. Increased destruction of red cells with haemoglobin breakdown.
2. Bone marrow regeneration.

Site of destruction is determined by

In intravascular destruction, there is release of free haemoglobin due to destruction of RBCs in the circulation.

In extravascular haemolysis, there will be removal of senescent RBCs from reticulo-endothelial cells (Fig. 1.30). Haemoglobin is released and catabolised within the macrophages. Indirect bilirubin may be increased but free haemoglobin is not detected in the plasma.

The aetiology is established by

- Clinical features
- Special investigations.

General Aspects

Age: Neonatal period H/O hyperbilirubinaemia

- Isoimmunisation
- Congenital haemolytic anaemia (HS, G6PD deficiency)
- Congenital infection.

3–6 months period H/O hyperbilirubinaemia

- Congenital disorder of haemoglobin synthesis
- Defects in haemoglobin structure.

Gender: X-linked disorders—G6PD deficiency, PK deficiency.

Race

Haemoglobin S and C—blacks
β-thalassaemias—whites
α-thalassaemias—black and yellow races.

Ethnicity

Thalassaemias—Mediterranean origin
G6PD deficiency—Jews, Greeks, Filipinos.

Infection

Infection induced HA (usually non-immune—malaria, babesiosis, *C. perfringens*).

Inheritance

Family history of anaemia, jaundice, gallstones, splenomegaly.

General Physical Examination

Skin: Jaundice, petechiae, purpura

Cavernous haemangioma, pregnancy (HELLP syndrome—haemolysis, elevated liver enzymes and low platelet count), microangiopathic HA.

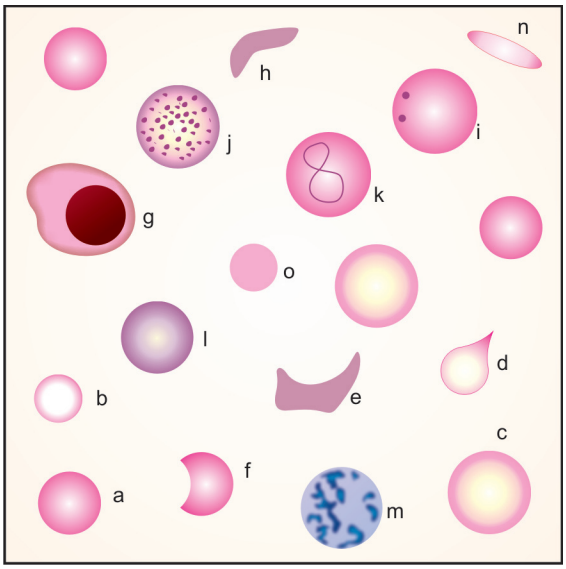


Fig. 1.30: Various red blood cells (poikilocytes): (a) Normal RBC, (b) microcyte, (c) macrocyte, (d) tear drop cell, (e) schistocyte, (f) bite cell, (g) nucleated RBC, (h) sickle cell, (i) Howell-Jolly body, (j) basophilic stipling, (k) Cabot ring, (l) polychromatophilic cell, (m) reticulocyte, (n) pencil-shaped cell, (o) spherocyte

Ulcers on lower limbs: S and C haemoglobinopathies, thalassaemias, sickle cell anaemia.

Facies and bones: Frontal bossing, prominence of molar and maxillary bones, thinning of cortical bone, spontaneous fractures, hand-foot syndrome.

Extra-medullary haematopoiesis

Eyes

Tortuosity of conjunctival and retinal vessels: HbS and HbC.

Microaneurysm of retinal vessels: S and C haemoglobinopathies.

Cataracts: G6PD deficiency, galactosaemia with HA in newborns.

Vitreous haemorrhage: S haemoglobinopathy.

Spleen and liver: Enlargement seen in most HAs.

Gallbladder: Stones (chronic hemolysis, congenital haemolytic anaemias).

LABORATORY EVIDENCE OF HAEMOLYSIS IN HAEMOLYTIC ANAEMIA

Thalassaemia

Thalassaemia was first recognised by Thomas B. Cooley. It is originally described in Italians, Greeks, and people of Mediterranean region. It also occurs in people of Middle East countries, South-East Asia and India.

It is a genetically determined disorder with autosomal dominant inheritance. There will be reduction in the rate of synthesis of normal haemoglobin polypeptide chains. Thus, there is less amount of adult haemoglobin (HbA) (Table 1.4).

Classification

Normally α and β chains are produced under separate genetic control and in normal state the synthesis is balanced.

There are two main groups of thalassaemia, one affecting synthesis of alpha chains is α thalassaemia and the other affecting beta chains is β thalassaemia.

Pathogenesis

In β thalassaemia there is less amount of HbA. There is production of gamma and delta chains, thus there is increased production of HbF and HbA2.

Due to lack of β chains the α chains accumulate, aggregate and interfere in erythroid cell maturation and function, resulting in premature destruction of RBCs.

In α thalassaemia the levels of HbA, HbF and HbA2 are reduced. The beta and gamma chains accumulate and form HbH (β4) and Hb Bart (γ4).

α thalassaemia and β thalassaemia are inherited co-dominantly and have homozygous and heterozygous states.

Clinical Features

Occurs in two forms: β thalassaemia major and β thalassaemia minor.

β thalassaemia major also called Cooley's anaemia is usually a severe illness characterized by total suppression of β chains. β thalassaemia minor or trait is mild form (Fig. 1.31).

If the severity falls in between the two, it is thalassaemia intermedia. These do not require transfusions or may require sporadically.

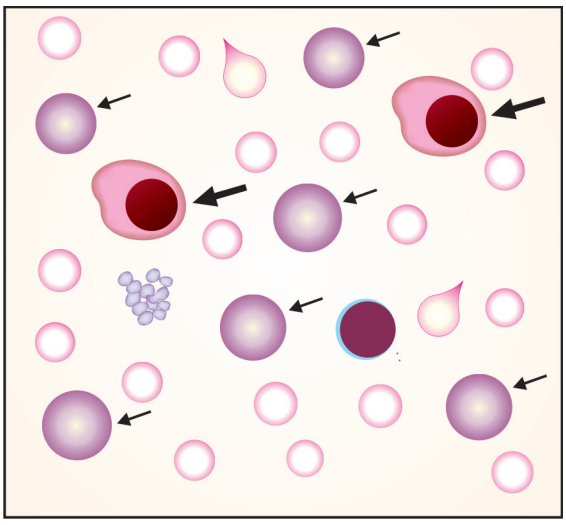


Fig. 1.31: Peripheral smear in thalassaemia major (thick arrow—nucleated RBCs, thin arrow—polychromatophilic cells)

Table 1.4: To show evidences for haemolysis

Evidence for increased red cell destruction
• Jaundice and hyperbilirubinaemia
• Reduced plasma haptoglobin (<250 mg/L) and haemopexin
• Increased plasma LDH (up to 800 IU/L) (N = 207 IU/L)
• Evidences of intravascular haemolysis
– Haemoglobinaemia
– Haemoglobinuria
– Methaemoglobinaemia/methalbuminaemia
– Increased urine and faecal urobilinogen
– Decreased glycosylated haemoglobin
• Evidences of extravascular haemolysis
– Positive Coombs' test
– Splenomegaly
Evidence for compensatory erythroid hyperplasia
Peripheral smear
• Reduced haemoglobin
• Elevated reticulocyte count—marked polychromasia
• Nucleated RBCs
Bone marrow
• Erythroid hyperplasia
• Reduced M/E ratio
Radiological changes
• Deforming changes in the skull and long bones—frontal bossing
Evidences of red cell damage
• Spherocytosis—HS, immune HA
• Increased red cell fragility
• Fragmented RBCs
• Schistocytes—mechanical damage
• Heinz bodies, bite/blister cells
• Compensated erythroid hyperplasia
• *Compensated haemolytic state:* A state of haemolysis in which the resulting increased erythrocyte production is able to keep up with accelerated RBC destruction, thus preventing development of anaemia.
• Reticulocytosis
• Macrocytosis/polychromasia
• Nucleated RBCs in peripheral blood
• Leukocytosis
• Normoblastic erythroid hyperplasia—bone marrow
Reduced red cell lifespan
Measurement of red cell survival no longer routinely done—Cr 51 (N $T_{1/2}$ = 25–35 days)

Thalassaemia major manifests by first year of life. The anaemia is insidious. With regular blood transfusions the child can have normal growth and development.

Inadequately Transfused Child

- Retarded growth and development
- Anaemia—weakness, lethargy, fever, appetite

- Changes in the skeletal system with mongoloid facies with thinning of cortical bone and pathological fractures
- Osteoporosis
- Extramedullary haemopoiesis can form masses and can compress the spinal cord
- Brown pigmentation of skin
- Hepatosplenomegaly
- Infections (functional hyposplenism), pericarditis due to streptococcal infection
- Gallstones
- Bleeding tendencies
- Secondary leukopenia and thrombocytopenia
- Cardiac failure
- Recent years numerous reports of thrombotic complications—possibly procoagulant phospholipids are exposed on RBCs and platelets and haemostatic system is activated. Also endothelial injury and iron overload are possible pathological mechanisms.

The consequences of repeated transfusions like iron accumulation in liver, heart, pancreas, etc., haemochromatosis with organ dysfunction can develop and death is usually by 2–3 decades. Pancreatic haemosiderosis can lead to diabetes and cirrhosis develops with deposition of iron in liver. Cardiac haemosiderosis leads to arrhythmias, heart block and chronic congestive heart failure.

Bone Changes

- Hyperplastic marrow
- Frontal bossing, maxillary hypertrophy
- Hair-on-end appearance of skull on X-ray.

Lab Findings

Peripheral smear (Fig. 1.31)

- Microcytic hypochromic anaemia (Hb of 3–9 g/dl), anaemia is severe
- Anisopoikilocytosis
- Nucleated RBCs
- Polychromasia (reticulocytes increased)
- Schistocytes, dacrocytes, ovalocytes, target cells
- Basophilic stippling.

Other findings

- Decreased MCV, MCH, MCHC, PCV
- Decreased osmotic fragility
- Increased serum uric acid
- Normal free RBC protoporphyrin.

Bone Marrow

- Normoblastic erythroid hyperplasia
- Increased macrophages
- Inclusion bodies in normoblast—methyl violet
- Prussian blue stain—abundance of iron.

Lab Findings-Hb Electrophoresis

The following procedures can be done.

- Citrate agar electrophoresis at alkaline or acid pH
- Capillary electrophoresis
- Automated high performance liquid chromatography
- Isoelectric focussing
- Globin chain electrophoresis

This is done to establish which globin chain is affected.

1. α-migrate towards cathode
2. β-migrate towards anode.

Hereditary Spherocytosis (HS)

- Autosomal dominant
- Primary membrane skeletal disorder of vertical protein interaction
- Defective or absent spectrin molecule, protein 4.2, ankyrin and band-3 protein
- HS—most commonly deficient spectrin and ankyrin
- Membrane instability—membrane loss.

Laboratory Findings

- Moderate/mild/no anaemia
- Reticulocytosis (5–20%)
- Nucleated RBCs

- The peripheral blood smear shows characteristic micro-spherocytes, which appear small, dark, round with no central pallor and decreased diameter
- Polychromasia
- Normal/decreased MCV
- Increased MCHC—hyperhaemoglobin
- Hyperbilirubinaemia
- Negative antiglobulin test
- Increased osmotic fragility
- Mild cases with incubation, OF increased. Defibrinated blood to be used for this test. Blood incubated for 24 hours at 37°C. Normal RBCs also show increased fragility on incubation due to swelling. HS cells lose membranes more readily than normal RBCs when incubated. This test has increased sensitivity and is the most reliable diagnostic test for HS
- Autohaemolysis
- Cryohaemolysis.

RETICULOCYTE COUNT (Fig. 1.32)

This count is one of the important investigations in diagnostic haematology. It must be remembered that the reticulocytes are juvenile red cells (Fig. 1.32). They contain remains of ribosomes and ribonucleic acids which are present in large amounts in nucleated precursors. Ribosomes and RNA material react with certain dyes such as brilliant cresyl blue and new methylene blue to form a blue precipitate of granules or filaments. This reaction takes place in supravital stains. In Romanowsky stained smears the reticulocytes take up diffusely basophilic tint. Most immature reticulocytes have the largest amount of granules and filaments, whereas less immature cells have least granules and filaments.

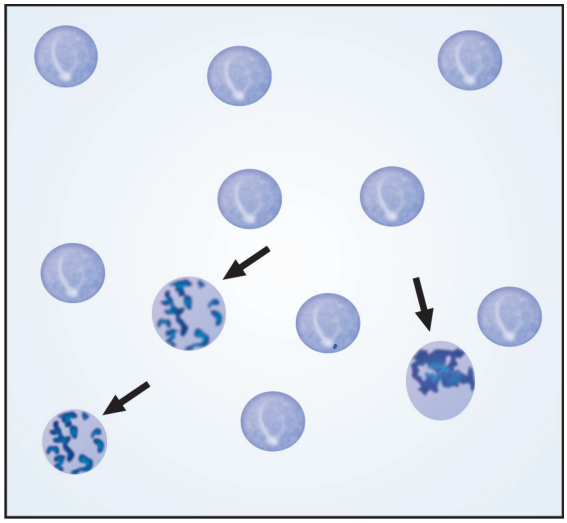


Fig 1.32: Reticulocytes (arrows)

The number of reticulocytes reflects the erythropoietic activity. After the cells have been released from the bone marrow, within one day they mature into RBCs. In some cases increased erythopoietin stimuli results in premature release of reticulocytes with longer time of maturation in circulation. In such cases reticulocyte maturation time and corrected reticulocyte count are to be deduced by using plasma iron turnover data.

Technique of Reticulocyte Count

1% brilliant cresyl blue

Brilliant cresyl blue	1.0 g
Sodium chloride	0.7 g
Sodium citrate	0.6 g
Distilled water	100 ml

New methylene blue can also be used instead of brilliant cresyl blue. New methylene blue stains reticulum filaments more deeply and more uniformly than the brilliant cresyl blue. New methylene blue is different from methylene blue; the latter is a poor reticulocyte stain.

Normal range

Adults and children	0.2 to 2.0%
Infants	2 to 6%

Note: Reticulocyte should be differentiated from

1. Pappenheimer bodies which are usually single and less commonly multiple.
2. HbH undergoes denaturation with brilliant cresyl blue or even with new methylene blue.

3. Heinz bodies are stained lighter than reticulocytes with new methylene blue stain.

Reticulocytes also can be counted employing fluorescent microscopy. In that case, one volume of acridine orange to one volume of blood is mixed for 2 minutes, and then make smears and observe under fluorescent microscopy.

Procedure for demonstration of reticulocytes

2–3 drops of new methylene blue and equal drops of blood are added to 75 × 10 mm glass or plastic tube. After mixing well keep at 37°C in a incubator for 15–20 minutes. Mix well again before preparing smears; smears should be well spread and the cells should be well stained. Interpret under oil immersion.

Counting of Reticulocytes

Adjustable diaphragms, paper or cardboard diaphragms could be used for counting the reticulocytes. In paper or cardboard diaphragms circle/square is cut and inserted in the eyepiece. RBCs counted with this diaphragm should be roughly 50. Such 20 fields are observed, so that roughly 1000 RBCs are inspected. In all these 20 fields, the reticulocytes ('n' cells) are counted.

Calculation

In 1000 RBCs = 'n' reticulocytes

For 100 RBCs = 100 × n/1000

The result is expressed in percentage.

Example: In 20 fields (1000 RBCs) 20 reticulocytes are counted.

Hence reticulocytes count is: (100 × 20)/1000 = 2%.

Corrected Reticulocyte Count

Counting of circulating reticulocytes is the simplest and very reliable sign of accelerated erythrocyte production.

The percentage of reticulocytes can increase either because there are more reticulocytes in the circulation or because there are fewer mature cells. In anaemias, however some prefer to correct the reticulocyte count by multiplying the percentage of reticulocytes by patient's haematocrit and then dividing the result by normal haematocrit.

Corrected reticulocyte count = Reticulocyte percentage × Patient's haematocrit/0.45

However corrected counts are not the perfect indices of production, as the percentage of reticulocytes could be altered by premature release from the marrow (shift). A reticulocyte production index (RPI) has been proposed to correct this shift.

RPI = Corrected reticulocyte count/2 (maturation time correction)

SICKLING PHENOMENON (Fig. 1.33)

This test detects the presence of HbS; because of the decreased solubility of the abnormal haemoglobin at low oxygen tension.

Methods: The two methods followed are as under.

1. Mix equal volume of blood and freshly prepared 2% sodium metabisulphite (0.2 g in 10 ml distilled water) on a slide. Place a cover slip. Seal the coverslip edges with vaseline or paraffin wax. Inspect for the resulting sickling under low power.

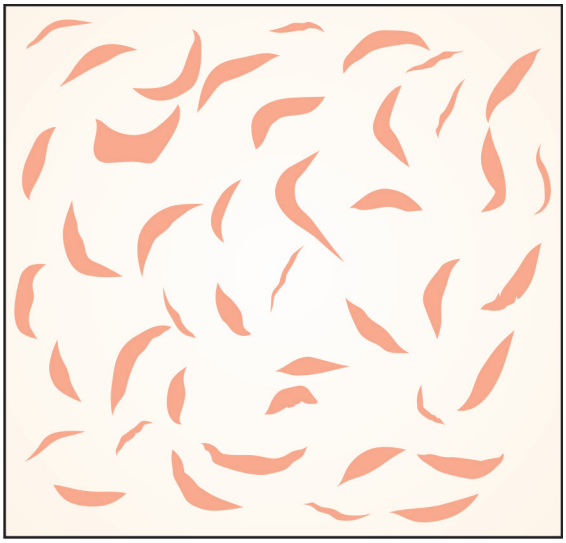


Fig 1.33: Schematic diagram of sickling

2. Two volumes of 0.114 M-sodium dithionite ($Na_2S_2O_4$) is mixed with three volumes of 0.114 M-disodium hydrogen phosphate (Na_2HPO_4) to give a final pH of 6.8. Sodium dithionite solution freshly prepared should be added to disodium hydrogen phosphate just before use. About 50 µl of the reagent is mixed with 10 µl of blood, then seal the sides of the coverslip and observed for sickling.

Sickling is visible immediately in HbS disease and within about 60 minutes in HbS trait. If this test is positive, then haemoglobin electrophoresis should be undertaken.

FOETAL HAEMOGLOBIN

1. Alkali Haematin Method

i. Take 10 drops of patient's blood and also the control blood in separate test tubes.
ii. Saline wash (2–3 changes) both of them.
iii. Add one and a half times (15 drops) of distilled water to the above, this is haemolysate.
iv. Add 1 ml of chloroform, mix well and centrifuge.
v. Take 3.2 ml of N/12 NaOH in a big test tube and 6.8 ml of precipitant reagent, i.e. 50% saturated ammonium sulphate in a small test tube.

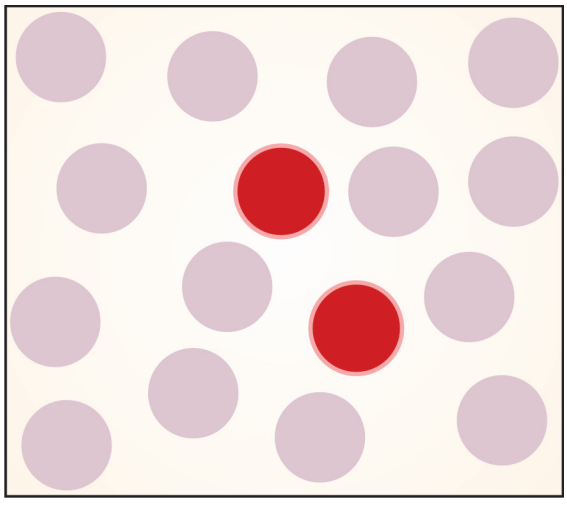


Fig. 1.34: Schematic diagram of cells with foetal Hb and normal cells as ghost cells

vi. Add 0.2 ml of haemolysate to 3.2 ml of NaOH; mix for 1 minute.
vii. After 1 minute add 6.8 ml of ammonium sulphate reagent to the above; mix by inverting.
viii. Filter, the filtrate contains undenatured HbF.
ix. Follow the same procedure for control.
x. For positive control, cord blood is used.

Interpretation

- Colourless—negative
- Pink colour—positive.

Note: KOH also can be used instead of NaOH

Acidified ammonium sulphate also can be used.

2. Acid Elution Method (Fig. 1.34)

Kleihaur, Braun and Betke in 1957 introduced this method. This method detects HbF containing cells; and their detection in maternal circulation has provided valuable information on the pathogenesis of haemolytic disease of the newborn. It must be noted that the HbF containing cells resist acid elution better than the normal cells. They appear as darkly staining cells amongst pale staining ghost cells. Occasional cells (reticulocytes) stain to an intermediate degree and are less easy to evaluate.

Fixative: 80% ethanol

Elution Solution

Solution A: 7.5 g/L haematoxylin in 96% ethanol.

Solution B: $FeCl_3$ 24 g, 25% HCl—20 ml, double distilled water to make 1 L.

For use, five volumes of solution A and one volume of solution B are mixed well. The pH is approximately 1.5. Once prepared the solution can be used for 4 weeks. If precipitate occurs, it should be filtered.

Counter stain: 1 g/L aqueous erythrocin or 25 g/L aqueous eosin.

Procedure

Air dry the smear, fix in 80% ethanol for 5 minutes in a coplin jar rapidly.

Then it should be rinsed in water and dried for 10 minutes. The slide is placed for 20 seconds in a coplin jar containing elution solution. Then the slide is washed thoroughly in water, placed for 2 minutes in eosin or erythrocin solution. Lastly, the slide is rinsed in tap water and dried.

Results: Cells with HbF—red, cells with HbA—pale pink (ghost cells)

OSMOTIC FRAGILITY (OF) (Fig. 1.35)

The rate of haemolysis is determined by the structure of the red cells. If the red cells are placed in 0.85% salt solution, the water neither enters nor leaves the cells. At lower concentrations of salt, the water enters the cells, eventually swells, ruptures and haemolyse the cells. When the rate of haemolysis is increased; the fragility of red cells is said to be increased. Similarly, when the rate of haemolysis is decreased, the fragility of the red cells is said to be decreased.

Methods: Following are the different methods to test for osmotic fragility.

Sanford method: Blood is added to graded series of 12 hypotonic salt solutions; the extent of haemolysis is noted after a period of 2 hours.

Dacie's method: Add heparinised blood to graded series of 12 hypotonic salt solutions buffered to pH of 7.4 and allow them to stand for 30 minutes. Centrifuge, read the degree of haemolysis spectrophotometrically and plot the percentage of haemolysis, against the percentage of salt concentrate.

Fragiligraph method: This method employs an electronic instrument.

Incubation method: In this method fibrinogen is removed; then incubate the defibrinated blood at 37°C for 24 hours, then follow the procedure of Dacie method.

OF increased

- Spherocytosis
- Acquired auto-immune haemolytic anaemia
- Erythroblastosis foetalis
- Burns
- Chemical poisons.

OF decreased

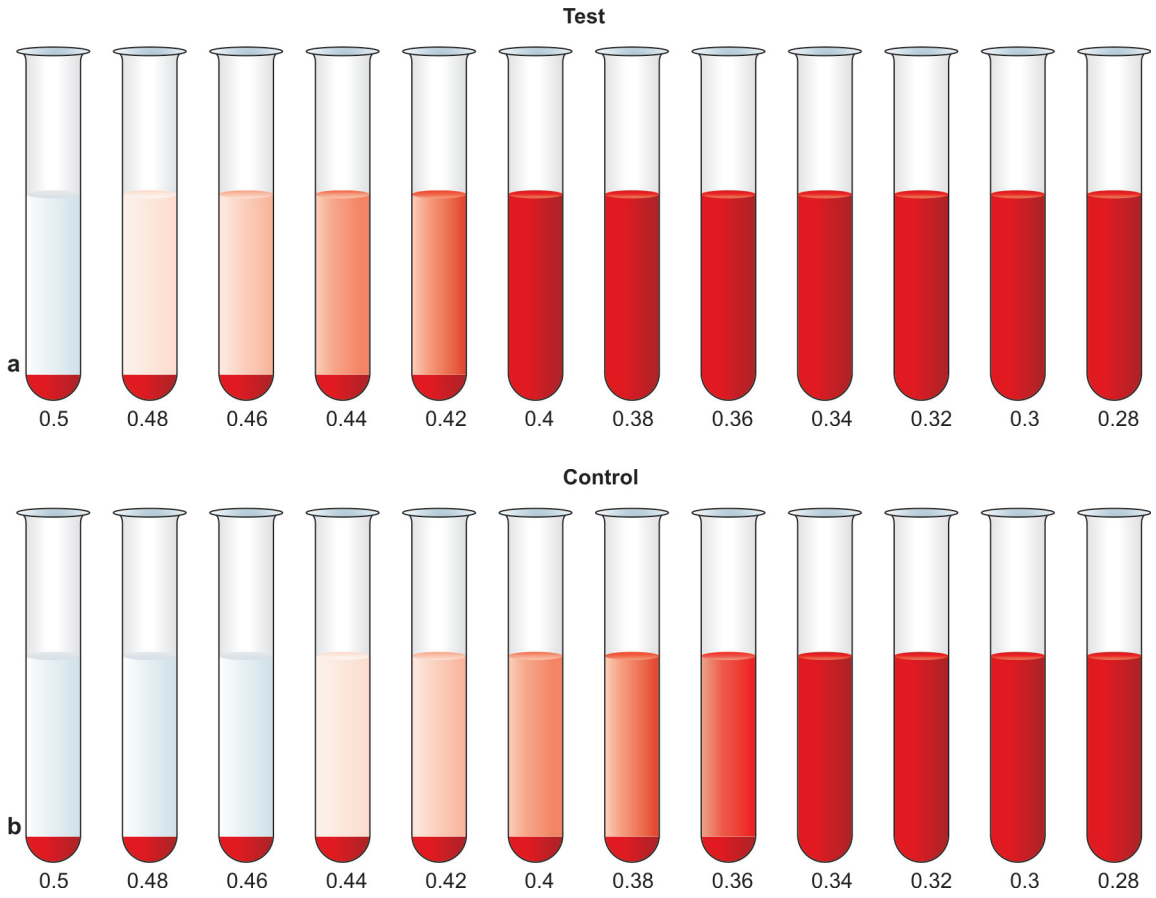
- Iron deficiency anaemia
- Thalassaemia major
- Sickle cell anaemia
- Obstructive jaundice
- Polycythemia vera
- Haemoglobin 'C' disease.

One set—test sample

No. of drops of 0.5% sodium chloride	25	24	23	22	21	20	19	18	17	16	15	14
No. of drops of distilled water	0	1	2	3	4	5	6	7	8	9	10	11
% of salt concentration obtained	0.5	0.48	0.46	0.44	0.42	0.4	0.38	0.36	0.34	0.32	0.30	0.28

Example:

	Haemolysis starts (Salt conc.)	*Haemolysis completed (Salt conc.)*	*Inference*
Control (normal)	0.44%	0.34%	Normal
Spherocytosis	0.48%	0.40%	OF increased
Sickle cell anaemia and thalassaemia	0.38%	0.30%	OF decreased



Figs 1.35a and b: Osmotic fragility

Sanford Method (Giffen and Sanford method)

The following reagents and equipment are required:

1. 0.5% sodium chloride
2. Distilled water
3. Two sets of Kahn test tubes: One set for control, and the other set for the test.
   One set has 12 test tubes in each row.

Procedure

Place one drop of blood in each tube.

Mix and allow to stand at room temperature for 2 hours.

Examine for the initial haemolysis and complete haemolysis.

Record the % of salt solution showing initial haemolysis and complete haemolysis. Compare the patient's results with the control results.

In control samples the initial haemolysis occurs at 0.44% or 0.42% of saline solution and completed in 0.34% saline solution.

Microcytic hypochromic cells are more resistant to haemolysis when compared to normal RBCs. Screening test for thalassaemia trait.

NESTROF Test (Necked eye single tube red cell osmotic fragility test)[2]

NESTROF test is performed using 0.36% buffered saline solution (Fig. 1.36). 2 ml of the solution is taken in two tubes and one is used for test and other as control. A drop of blood is added to each tube and they are left

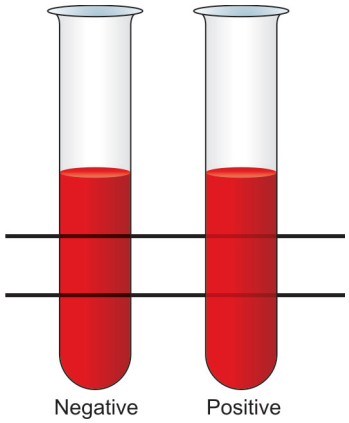


Fig.1.36: NESTROF

undisturbed for half an hour at room temperature. Both the tubes are then shaken and held against a white paper with a black line. The line is clearly visible through the contents of the tube containing control sample. If the line is not clearly visible, the test is considered positive. A positive test indicates lowered red cell osmotic fragility, and useful in detecting thalassaemia trait patients.

HAEMOGLOBIN ELECTROPHORESIS (Fig. 1.37)

1. Cellulose Acetate Electrophoresis at Alkaline pH

For routine work, electrophoresis at pH 8.4–8.6 using cellulose acetate membrane as a substrate is simple, rapid and sensitive.

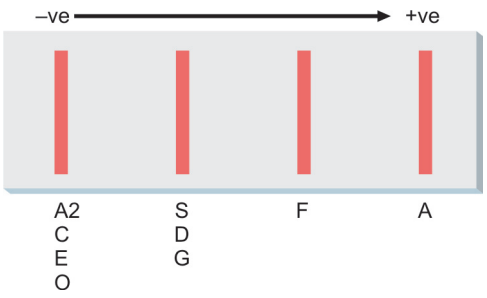


Fig. 1.37: Haemoglobin electrophoresis with separation of various haemoglobins

Principle

Electrophoresis is the movement of charged particles in an electrical field. At an alkaline pH, haemoglobin is a negatively charged protein. Therefore, the haemoglobin migrates towards the anode in an electric field. During electrophoresis, various haemoglobins separate because of differences in charges caused by structural variations of the haemoglobin molecule.

Specimen: EDTA or heparinised blood.

Equipment: Electrophoresis tank with power pack, wicks of filter or chromatography paper, blotting paper, applicators, cellulose acetate membrane, staining equipment, drying oven and pH metre.

Preparation of lysate for immediate use

Lyse one volume of washed red cells in 4 volumes of lysing agent. Such a lysate should not be kept for more than 1–2 days at 4°C as it forms gel.

Lysing agent

- 3.8 g EDTA, tetrasodium salt
- 0.7 g potassium cyanide
- Water to make 1 litre.

Note: There are different methods to prepare the lysate.

Reagents

Electrophoresis buffer: Tris/EDTA/borate (TEB) pH 8.5, Tris (hydroxymethyl) amino-methane 10.2 g, EDTA 0.6 g, boric acid 3.2 g and water to make 1 litre.

The buffer is stored at 4°C and can be used without any deterioration.

Protein stain: Ponceau S 5 g, trichloracetic acid 7.5 g and water to make 1 litre.

Destaining solution: 5% acetic acid—50 ml and water to make 1 litre.

Clearing solution: Glacial acetic acid 125 ml, methanol 375 ml and polyethylene glycol 20 ml.

Method

1. Prepare a lysate, further dilute the sample to 1 : 4 or 1 : 5 in water (to about 20 g/L).

2. Fill the electrophoresis tank with TEB buffer. Soak and position the wicks.
3. In a separate dish, soak the cellulose acetate membrane in TEB buffer for 5 minutes. Immerse the membrane slowly to avoid trapping of air bubbles.
4. Blot the membrane between two pieces of absorbent paper, but do not let it dry.
5. Place small volume (10 µl) of each diluted sample into a sample well.
6. Dip the applicator into the sample.
7. Apply the samples to the cellulose acetate approximately 3 cm from one end of membrane.
8. Place the membrane upside down across the bridge of the tank so that the cellulose acetate surface is in contact with the buffer, with the line of application at the cathode end.
9. Connect the power supply and run at 250–350 V for 20 minutes until a visible separation is obtained.
10. Disconnect the power supply, remove the membrane and stain with ponceau S for 3 to 5 minutes.
11. Remove the membrane, drain and elute the excess stain with three changes of destaining solution for 2 minutes each.
12. Dehydrate in methanol for 2 to 3 minutes.
13. Immerse in clearing solution for 4 to 6 minutes.
14. Dry at 65°C for 4 to 6 minutes.
15. Label and store the membrane in a protective plastic envelope.

Cellulose acetate electrophoresis is useful in the diagnosis of HbS and HbC haemoglobinopathies. It is also used to screen the elevated levels of HbA2 in thalassaemia trait.

Other methods

Citrate agar gel electrophoresis and acid gel method.

Globin chain synthesis rate studies: Peripheral blood incubated with radioactive labelled amino acid, which is then incorporated into the newly synthesised chains, which are separated by chromatography and their relative production is estimated by determining radioactivity.

- Reduced β to α chain ratio <0.25
- Mutation detection—PCR.

HbF estimation—alkali denaturation test

HbF resists alkali denaturation

- Washed RBCs → lysed + 1.2 N NaOH → HbA denatured, HbF resists → add ammonium sulphate → precipitation of HbA → filter → HbF left in filtrate → measure spectrophotometrically
- % HbF = HbF by alkali denaturation total Hb by cyanmethaemoglobin method
- Reference interval for adults is <2%.

Exercise

16

Leukaemias

Leukaemia is the clonal expansion of a single transformed stem cell resulting in accumulation of immature and non-functional haematopoietic cells in the bone marrow and body organs.

Aetiology and Leukaemogenesis

- Activation of proto-oncogene to oncogene—e.g. t(8;14) C-MYC to immunoglobulin, BALL, abnormal cellular proliferation
- Formation of chimeric transcription factor t(15;17) RAR/PML transcription repressors block differentiation—AML
- Formation of fusion protein with enhanced tyrosine kinase activity—t(9;22) BCR/ABL enhanced tyrosine kinase activity
- Inactivation of tumour suppressor gene pathway—RB1 p53.

Leukaemias are broadly classified as acute or chronic depending upon age of onset, course of disease and clinical presentation. Comparison between acute and chronic leukaemia is given in Table 1.5.

ACUTE LEUKAEMIAS

Definition

These are stem cell disorders characterised by malignant neoplastic proliferation of a transformed cell. Classic triad of acute leukaemia is anaemia, infections and bleeding. Two major categories are:

1. Acute myeloid leukaemia (AML) or acute non-lymphoid leukaemia.
2. Acute lymphoblastic leukaemia (ALL).

Presentation

Age: Acute leukaemia may occur at any age. ALL is common during 2–10 years of age.
Symptoms: Fatigue, pallor, fever, weight loss, bone pains.
Signs: Hepatosplenomegaly, lymphadenopathy, anaemia, neutropaenia, thrombocytopaenia

Table 1.5: Comparison of acute and chronic leukaemias

	Acute	*Chronic*
Age	All ages	Adults
Clinical onset	Sudden	Insidious
Course of disease	Weeks to months	Months to years
Predominant cells	Blasts and few mature forms	Mature forms
Anaemia	Mild to severe	Mild
Thrombocytopaenia	Mild to severe	Mild
WBC count	Variable	Increased

General Laboratory Findings

Peripheral smear: Leukocyte count usually increased but may be normal or decreased. There can be presence of lymphoblasts or myeloblasts in the peripheral blood. Platelets are usually reduced.

Bone marrow: Hypercellular with lymphoblasts or myeloblasts equal to or >20%.

Other Investigations of Leukaemias

- Hyperuricaemia and increased LDH (increased cell turnover)
- Impairment of renal function (leukaemic infiltration)
- CNS—frequent site for extramedullary spread, CSF should be analysed for presence of blasts
- Cytochemistry
- Flow cytometry
- Cytogenetics.

Classification of ALL (FAB classification)

L1 : Small, homogenous blasts, scanty cytoplasm, indistinct nucleoli.

L2 : Large, heterogeneous blasts, indented nuclei, one or more nucleoli, abundant cytoplasm, minimal cytoplasmic vacuolation.

L3 : Large, homogenous blasts, abundant basophilic cytoplasm with prominent cytoplasmic vacuolations (Burkitt).

Acute Myeloid Leukaemia (Figs 1.38a to c)

The defect primarily affects the common myeloid progenitor (CMP) cell.

Myeloblasts in peripheral blood or bone marrow should be >20% (WHO, 2001).

According to the FAB classification of myeloblasts in peripheral blood or bone marrow should be >30% (Table 1.6).

Table 1.6: FAB classification acute myeloid leukaemia

	Morphology		*Myeloperoxidase (MPO)*	*Sudan black B (SBB)*
M0	Acute myeloblastic leukaemia (AML): Minimally differentiate	>30% blasts; no granules	–ve	–ve
M1	AML with no maturation	>30% blasts, few granules +/– Auer rods	+ve	+ve
M2	AML with maturation	>30% blasts, granules common, + Auer rods	+ve	+ve
M3	Acute promyelocytic leukaemia	>30% blasts, prominent granules, ++ Auer rods	++	++
M4	Acute myelomonoblastic leukaemia	>30% blasts, >20% monocytes, + Auer rods	+	+
M4eos	Acute myelomonocytic leukaemia with eosinophilia	>30% blasts, >20% monocytes, > 5% abnormal eosinophils, + Auer rods	+	+
M5 a/b	Acute monblastic leukaemia with or without maturation	>30% blasts, >80% monoblasts with or without maturation	+	+
M6	Acute erythroleukaemia	>30% myeloblasts, >50% erythroblasts, + Auer rods	+(myeloblasts)	+(myeloblasts)
M7	Acute megakaryocytic leukaemia	>30% megakaryoblasts, cytoplastic budding +	–	–

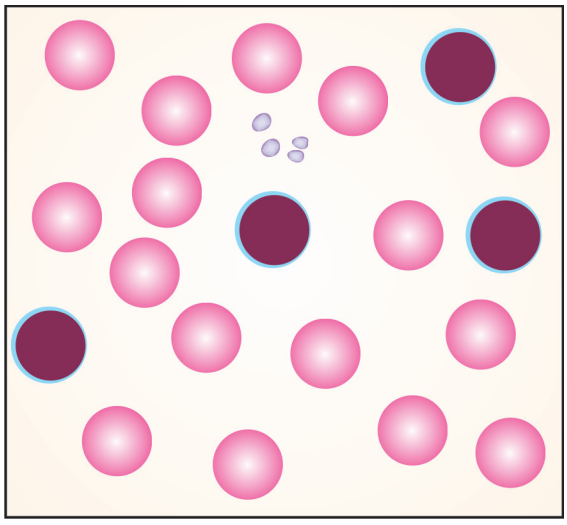


Fig. 1.38a: Peripheral smear in acute leukaemia (ALL—L1)

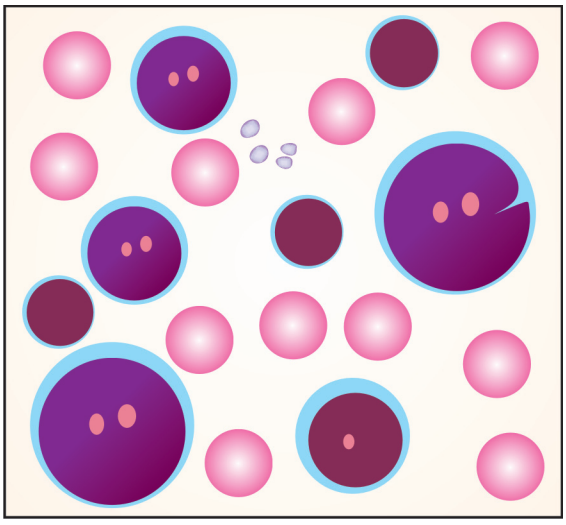


Fig. 1.38b: Peripheral smear in acute leukaemia (ALL—L2)

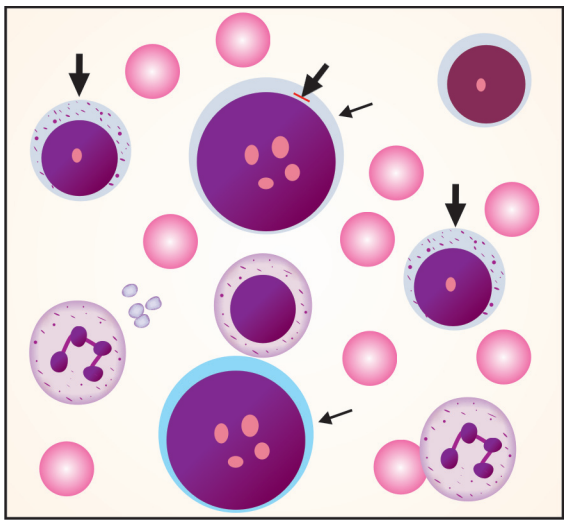


Fig. 1.38c: Peripheral smear in acute leukaemia (AML), note myeloblasts (one myeloblast with Auer rod) and promyelocytes (thin arrow—myeloblast, thick arrow—promyelocyte)

Modified 2016 WHO Classification[3]

I. *AML with Recurrent Genetic Abnormalities*

- AML with t(8;21) (q22;q22); RUNX1-RUNX1T1
- AML with inv(16)(p13;q22)or t(16;16)(p13.q22),CBFB/MYH11
- APML with t(15;17)(q22;12), PML-RARA and variants
- AML with t(9;11)(p22;q23);MLLT3-MLL
- AML with t(6;9)(p23;q34);DEK-NUP214
- AML with inv(3)(q21q26.2); RPN1-EVI1
- AML (megakaryoblastic) with t(1;22)(p13;q13); RBM15-MKL1
- AML with BCR-ABL (Provisional)
- AML with mutated NPM1
- AML with mutated CEBPA
- AML with mutated RUNX1

II. *AML with Myelodysplasia Related Changes*
III. *Therapy Related Myeloid Neoplasms*
IV. *AML-NOS*
- AML with minimal differentiation
- AML without maturation
- AML with maturation
- Acute myelo monocytic leukemia
- Acute monoblastic leukemia
- Pure erythroid leukemia
- Acute megakaryocytic leukemia
- Acute basophilic leukemia
- Acute panmyelosis with myelofibrosis

V. *Myeloid Sarcoma*
VI. *Myeloid Proliferations Related to Down Syndrome*
VII. *Blastic Plasmacytoid Dendritic Cell Neoplasms*
VIII. *Acute Leukemia of Ambiguous Lineage*
IX. *B-Lymphoblastic Leukemia/Lymphoma*
X. *T-Lymphoblastic Leukemia/Lymphoma*

General Laboratory Findings in AML

Peripheral Blood

WBCs:
Total count—elevated, may exceed 1 lakh/cmm.

50% of the cases may have normal or decreased counts at the time of presentation.

Differential count shows presence of myeloblasts (WHO > 20%).

Myeloblast in AML—typically 20 μm in diameter. Nucleus composed of dispersed chromatin and has 3–4 prominent nucleoli. Cytoplasm may show Auer rod.

RBCs: Decreased in number.

Platelets: Reduced, hypogranular and occasional giant platelets may be present.

Buffy coat smear—undertaken if strong suspicion of AML but no blasts in the peripheral smear.

Bone Marrow Examination

It is typically—hypercellular with predominance of blasts (≥20).

FAB group classified neoplastic myeloblasts as:
- Type I—Blasts without any granules
- Type II—Blasts with <20 granules
- Type III—Blasts with numerous granules.

CHRONIC MYELOID LEUKAEMIA (CML)

Definition

It is a clonal stem cell disorder characterised by the acquisition of an oncogenic BCR/ABL fusion protein [usually the result of a reciprocal translocation (9;22)(q34;q11)] and by proliferation of granulocytic elements at all stages of differentiation.
- t(9;22) is also referred to as the Philadelphia chromosome
- Average incidence of CML 45 years
- Men > women.

Three Clinical Phases

- Chronic phase
- Accelerated phase
- Blast crisis

Most patients are diagnosed while still in the chronic phase.

Chronic Phase

- <10% are myeloblasts, platelets tend to be normal or increased in number low to absent.
- Increased percentage of myelocytes
- Low leukocyte alkaline phosphatase activity (low LAP score).
- There is basophilia.

Accelerated Phase

- Myeloblasts—10–19% in PS or BM
- Basophils more than 20%
- Persistent thrombocytopaenia
- Increasing spleen size and counts in spite of therapy
- Cytogenetic evidence clonal evolution.

Blast Phase

- Blasts ≥20%
- Extramedullary blast proliferation
- Large aggregates/clusters of blasts in the bone marrow.

Clinical features indicating a more difficult-to-control marrow proliferative state are suggestive of progression. These include:

- Rapidly rising WBC count that is more refractory to treatment
- Increasing splenomegaly
- Fever, bone pain, and weight loss
- Laboratory features include more immature cells in the peripheral blood or marrow
- Increasing eosinophils or basophils
- The appearance of more chromosome anomalies, including additional Philadelphia chromosomes.

Diagnostic Approach to CML (Figs 1.39a to d)

Peripheral blood smear and marrow biopsy.

Ph+ chromosome by karyotypic analysis or the presence of the BCR-ABL translocation by Southern blot or polymerase chain reaction (PCR) assays confirms the diagnosis.

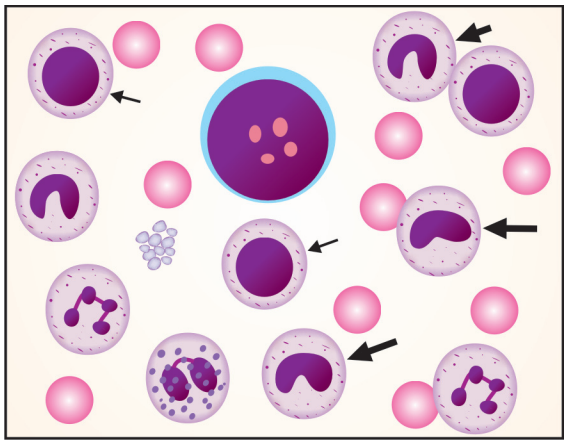


Fig. 1.39a: Schematic diagram of peripheral smear in CML (thin arrow—myelocytes, thick arrow—metamyelocytes, short arrow—band forms)

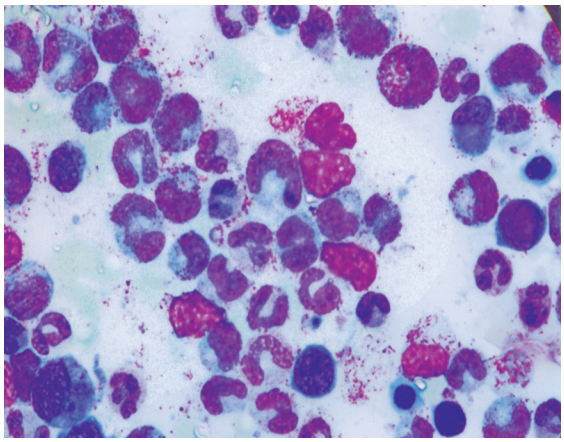


Fig. 1.39b: Bone marrow in CML

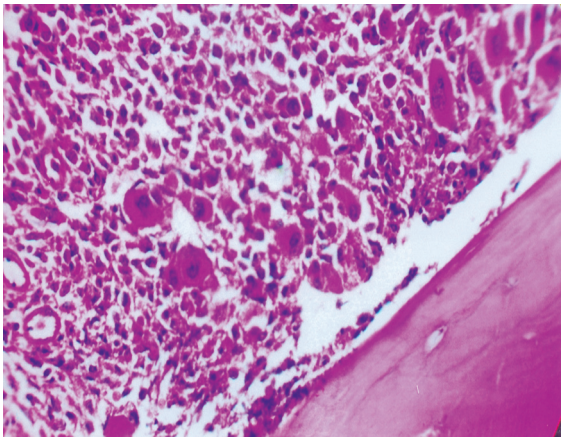


Fig. 1.39c: Trephine biopsy in CML (note plenty of megakaryocytes)

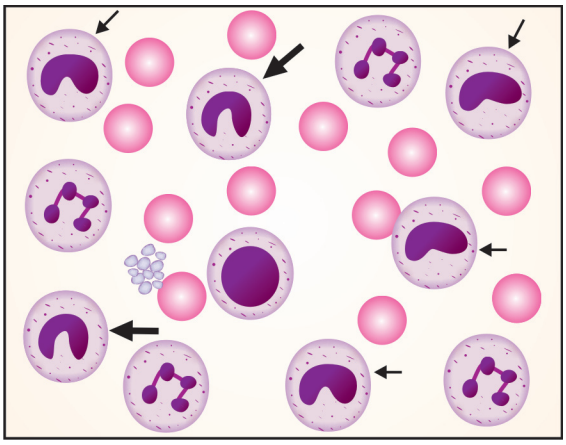


Fig. 1.39d: Schematic diagram, leukaemoid reaction (note metamyelocytes—thin arrow, band forms—thick arrow and increased neutrophils)

Peripheral Blood

1. There is a predominance of mature neutrophils.
2. Basophils are increased in number.
3. Increased percentage of myelocytes (so-called myelocyte bulge).
4. <10% are myeloblast.
5. Many patients may also demonstrate eosinophilia.
6. Platelets tend to be normal or increased in number.
7. Low to absent leukocyte alkaline phosphatase activity (low LAP score).

Differential Diagnosis (WHO 2008 classification of MDS/myeloproliferative diseases)

1. Chronic myelomonocytic leukaemia (CMML)
2. Juvenile myelomonocytic leukaemia (JMML)
3. Atypical CML
4. MDS/myeloproliferative diseases, unclassifiable.

The differences between chronic myeloid leukaemia and leukaemoid reaction are shown in Table 1.7.

CHRONIC LYMPHOCYTIC LEUKAEMIA

Chronic lymphocytic leukaemia (CLL) is characterized by the accumulation of mature-appearing lymphocytes in the blood, marrow, lymph nodes, and spleen.

The CLL cells are monoclonal B lymphocytes that express CD19, CD5, and CD23, with weak or no expression of surface immunoglobulin (Ig), CD20, CD79b, and FMC7.

Clinical Findings

1. CLL occurs in elderly people usually in more than 60 years of age.
2. About 70 to 80% of the patients are diagnosed incidentally.
3. Lymphadenopathy and or splenomegaly may be detected during a routine physical examination.
4. Less frequently, enlarged nodes or the development of infection is the initial complaint.
5. Fever and weight loss are uncommon at presentation but may occur with advanced stage.
6. Enlargement of the cervical and supraclavicular nodes occurs more frequently

Table 1.7: Differences between chronic myeloid leukaemia and leukaemoid reaction

Laboratory parameter	*CML*	*Leukaemoid reaction*
Leukocytes	Blasts and promyelocyte in peripheral blood; toxic changes usually absent; eosinophilia and basophilia; neutrophila with single lobed nuclei and hypogranular forms may be present	Toxic granulation; Dohle bodies and vacuoles present; blasts and promyelocytes rare; no absolute basophilia or eosinophilia
Platelets	Often increased with abnormal morphological forms present; occasional micromegakaryocytes	Usually normal
Erythrocytes	Anaemia usually present; variable anisocytosis; poikilocytosis; NRBC present	Anaemia may be present, but NRBC not typical
LAP	Low	Increased
Chromosome karyotype	Ph chromosome or BCR/ABL translocation present	Normal

than axillary or inguinal lymphadenopathy. The lymph nodes are usually discrete, freely movable, and non-tender.

7. Usually mild to moderate enlargement of the spleen is present.
8. Enlarged tonsils and mesenteric or retroperitoneal lymphadenopathy is less common.
9. Anaemia and thrombocytopaenia occur in later stages.
10. CLL patients may present with autoimmune hemolytic anaemia (AIHA).

Prognosis depends upon the stages of Binet and modified Rai clinical staging:

Binet Staging System for CLL

Stage	*Description*
A	≤2 lymphoid bearing areas enlarged
B	≥3 lymphoid bearing areas enlarged
C	Presence of anaemia (Hb <10 g/dl) or thrombocytopaenia (platelet count <100,000/L).

Five lymphoid bearing areas are cervical, axillary, inguinofemoral, spleen and liver.

Modified Rai Clinical Staging for Chronic Lymphocytic Leukaemia

Risk	*Stage*	*Description*
Low	0	Lymphocytosis in blood and bone marrow
Intermediate	I	Lymphocytosis + enlarged lymph nodes
	II	Lymphocytosis + enlarged liver or spleen with or without lymph nodes
High	III	Lymphocytosis + Anaemia (Hb <11 g/dl) with or without enlarged liver, spleen or lymph nodes
	IV	Lymphocytosis + thrombocytopaenia (platelet count <1,00,000/cu mm) with or without anaemia or enlarged liver, spleen or lymph nodes

Peripheral Blood and Bone Marrow (Figs 1.40a and b)

- In most patients, there is increased number of mature lymphocytes in the peripheral blood and bone marrow. These cells have the morphologic appearance of normal

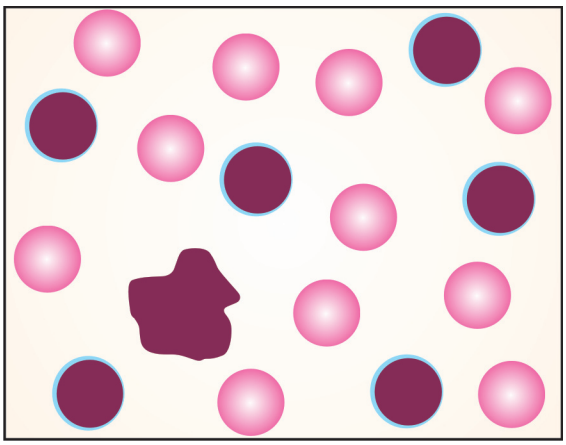


Fig.1.40a: Peripheral smear in CLL. Note increased number of mature lymphocytes and a smudge cell

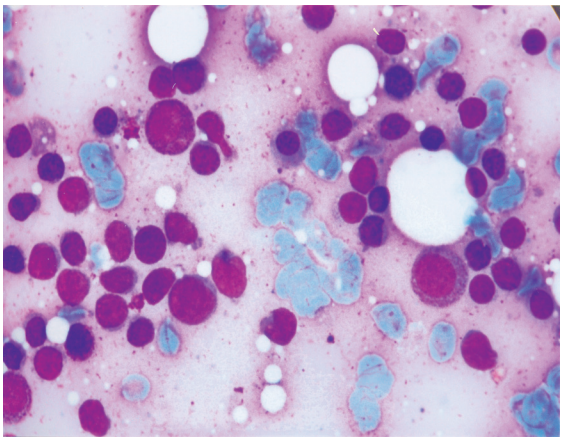


Fig. 1.40b: Bone marrow in CLL

small to medium-sized lymphocytes with clumped chromatin, inconspicuous nucleoli, and scant cytoplasm.

- Smudge cells (basket cells or shadow cells of Gumprecht) are commonly seen in the blood smear.
- In classical CLL, >90% of the cells are mature lymphocytes.

When 11 to 54% of the cells are prolymphocytes, it is termed CLL/PLL.

- If ≥55% of the cells are prolymphocytes, it is termed prolymphocytic leukaemia.

When >15% of the lymphocytes are plasmoid or cleaved and <10% are prolymphocytes, it is termed atypical CLL.

Exercise

17

Multiple Myeloma/Plasma Cell Dyscrasias

Plasma Cell Dyscrasias Constitute the Following:

Malignant proliferation

- Multiple myeloma (MM)
- Waldenström macroglobulinaemia (WM)
- Plasmacytoma
- Heavy chain disease.

Relatively benign

- Monoclonal gammopathy of undetermined significance (MGUS)
- Smoldering MM
- Primary systemic amyloidosis
- POEMS syndrome (polyneuropathy, organomegaly, endocrinopathy, monoclonal gammapathy and skin changes).

Multiple Myeloma

Definition: Multiple myeloma is a malignant disorder characterised by proliferation of a single clone of plasma cells in the bone marrow. There will be lytic lesions of bones, increased monoclonal gammaglobulins and hypercalcemia (Table 1.10).

Etiology: Radiation exposure, exposure to benzene, smoking, alcohol and obesity has increased risk while diet with cruciferous vegetables, fish and vitamin C has reduced risk.

Pathogenesis: IL-6 plays role in proliferation of plasma cells and lytic lesions of bone.

The different diagnostic criteria are given in Tables 1.8 and 1.9.

Table 1.8: International myeloma working group criteria for diagnosis

1. M Protein in serum or urine
2. Clonal bone marrow plasma cells ≥10% or plasmacytoma biopsy proven
3. Myeloma related organ dysfunction—CRAB features
   a. Calcium elevation >11.5 mg%
   b. Renal insufficiency serum creatinine >1.96 mg%
   c. Anaemia <10 g/dl
   d. Bone lesions: Lytic/osteoporosis
4. In the absence of end organ damage, clonal plasma cells ≥60%
5. In updated International myeloma working group, myeloma is considered when CRAB features are present in a patient with smouldering MM.

CRAB features of MM: Calcium (elevated), renal failure, anaemia and bone lesions

Table 1.9: Revised criteria International myeloma working group 2014[4]

In asymptomatic patients following criteria can label the patient as MM:

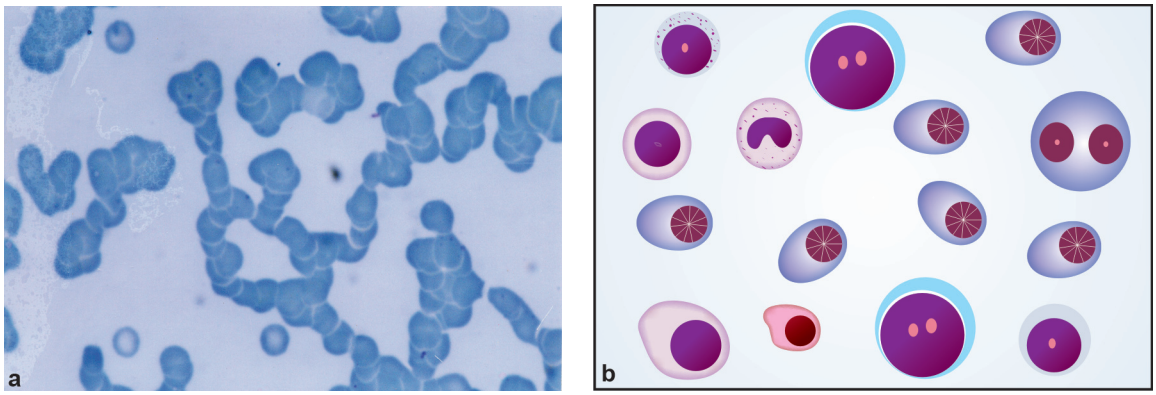
1. Clonal bone marrow plasma cells ≥60%.
2. Free light chain ratio more than 100.
3. MRI showing more than one focal lesion.

Table 1.10: Clinical presentation of multiple myeloma

Age: Old age (median age 65 years)	Elevated ESR markedly raised often >100 mm/hr
Insidious onset	Hypercalcaemia
Weakness, fatigue	BJ proteins
Pallor	Renal failure
Bone pains	Hyperviscocity: Hypergammaglobulinaemia
Pathological fractures	Amyloidosis
Recurrent infections	Marrow failure

Laboratory Evaluation

- CBC with peripheral smear
- ESR: Increased
- Bone marrow examination (Figs 1.41a and b)
- Chemistry panel (creatinine, Ca^{++}, LDH, 2M)
- Immunofixation electrophoresis
- Serum free light chain (FLC)
- Urinalysis/24 hours urine for protein
- Bence Jones protein
- Immunophenotype, cytogenetics
- Skeletal survey/CT/MRI/PET
- Plasma cell labelling index.



Figs 1.41a and b: (a) Peripheral smear, (b) bone marrow in multiple myeloma

Note: In peripheral smear Rouleaux formation and bone marrow with increased plasma cells.

Exercise

18

Bleeding Disorders

Bleeding disorders or haemorrhagic disorders can be due to any of the following:

- Vascular defects
- Platelet abnormalities
- Coagulation disorders.

VASCULAR CAUSES

These can be acquired or congenital

Acquired causes

- Simple easy bruising
- Senile purpura
- Non-thrombocytopenic purpura: Infections, drugs, uraemia, Cushing's disease, and adrenocorticosteroid administration
- Scurvy
- Dysproteinaemias
- *Miscellaneous disorders:* Orthostatic purpura, mechanical purpura, fat embolism, systemic disorders—collagen disease especially polyarteritis nodosa.

Congenital

Osler-Rendu-Weber disease, Ehlers-Danlos disease.

PLATELET CAUSES

These can be: Thrombocytopenia or functional defects.

Causes of thrombocytopenia: Acquired or congenital.

Acquired causes: Common causes are:

- **Idiopathic thrombocytopenic purpura** (ITP)—acute or chronic
- Drugs and chemicals
- Leukaemias
- Aplastic anaemias
- Bone marrow infiltration
- Hypersplenism
- Disseminated lupus erythematosus.

Less common causes

- HIV infection
- Megaloblastic anaemia
- Liver disease
- Alcoholism
- Massive blood transfusion
- DIC
- Food allergy.

Functional defects

- Membrane receptor defects—Glanzmann's thrombasthenia, Bernard-Soulier syndrome
- Enzyme defects
- Granule defects

COAGULATION DISORDERS

- Haemophilia A and B
- von Willebrand's disease
- Other factor deficiency disorders—Factor I (fibrinogen), Factor II (prothrombin), Factor V, Factor VII, Factor X, Factor XI, Factor XII and Factor XIII.

Exercise

19

Tests Related to Bleeding Disorders

BLEEDING TIME

The time between blood oozing out of a vessel after injury until the arrest of haemorrhage is bleeding time (BT). In case of any vessel wall defects and platelet disorders the bleeding time is increased.

Ivy's Method

Tie the blood pressure apparatus to the arm and raise the pressure to 40 mm Hg. The area below the antecubital fossa is cleaned with 70% alcohol. Two separate punctures of 2.5 × 1 mm size, 5–10 cm apart are made using disposable lancet. Blot every 30 seconds. Record the time when bleeding stops. Take average of the two.

Normal range: 2–7 minutes.

Template Method (Modified Ivy's method)

This method is similar to Ivy's method. Raise the blood pressure to 40 mm Hg. Clean the antecubital fossa with 70% alcohol. With disposable template, make two standard cuts of 11 × 1 mm (length × depth). Blot every 30 seconds until bleeding stops. Take the average of the two.

Normal range: 2–10 minutes.

Duke's Method

Ear lobe is cleaned with 70% alcohol; 2.5 × 1 mm puncture is made with a lancet, start the stopwatch. Blot every 30 seconds. The time required for bleeding to cease is recorded.

Normal range: 2–7 minutes.

Note: Not a reliable method when compared to Ivy's method.

CLOTTING TIME

Clotting time (CT) is the time taken for the fluid blood to be converted into blood clot that is solid mass of platelets and fibrin.

CT increases in coagulation factor disorders and in such patients who are on anticoagulant treatment.

Capillary Tube Method

Prick the finger after aseptic precautions and simultaneously start the stopwatch as soon as the prick is given. Allow a drop of blood to collect at the prick site. Touch one end of the capillary tube to the blood collected. Blood enters the capillary tube by capillary action. At the end of 2½ minutes the capillary tube is gently broken into two pieces in the middle. Observe for fibrin thread. Repeat this thereafter every ½ minute and record the time taken for the fibrin thread formation.

- Normal range: 4–9 minutes

Note: It is not a reliable method.

Whole Blood Coagulation Time (Lee and White's method)

Take 3 test tubes each of 13 mm × 100 mm (2 or 4 test tubes also can be used). Start the stopwatch as soon as blood enters into the syringe. In each tube 1 ml of blood is taken.

3rd test tube will be the last to receive the blood. Place all the test tubes in a water bath at 37°C.

Every 30 seconds tilt the first test tube until the blood clots. Note the time of clotting. Similarly repeat the second and followed by the third test tube. Time of clotting is noted in each of the test tubes and then the average is calculated.

Normal range: 7–15 minutes

CAPILLARY RESISTANCE TEST (HESS/TOURNIQUET TEST)

Other names: Hess test, tourniquet test, cuff test, Rumpel-Leede test, Rumpel-Leede phenomenon.

Purpose

To determine the resistance of capillaries. In health, capillaries resist pressure of 100 mm Hg.

Procedure

Blood pressure cuff is tied to the arm. Blood pressure is raised to 100 mm Hg (80–100 mm Hg). Maintain the pressure for 5 minutes. After 15 minutes observe the forearm for petechiae. Count the number of petechiae.

Following inference is drawn:

Less than 10 petechiae	Negative/Normal
10–20 petechiae	Doubtful
> 20 petechiae	Positive

The test is positive in thrombocytopaenia and in von Willebrand's factor deficiency.

CLOT RETRACTION

Volume of serum expressed by the blood, which is allowed to clot at 37°C is recorded as a percentage of the original volume of blood.

Collect 5 ml of blood in a 10 ml centrifuging graduated test tube. Place a glass rod in vertical position in the tube. Incubate the tube at 37°C for 1 hour undisturbed. Normal clot retraction is 50–60%.

CLOT LYSIS TIME

After noting the clot retraction, continue to incubate the tube until 72 hours. The fibrin clot dissolves due to fibrinolysis and the red cells sink to the bottom. Normal clot lysis time is 72 hours. It is abnormal when the clot lysis occurs within 24 hours.

PROTHROMIN TIME (PT)

Clinical Significance

- PT reflects the overall efficiency of the extrinsic system
- Most sensitive to changes in factor V, VII, X
- Lesser to factor II and I.

Principle

Thrombokinase preparation containing calcium is added to citrated plasma. In presence of factor VII, extrinsic pathway is activated leading to clot formation.

Procedure

To 0.1 ml of patient's plasma add 0.1 ml of brain thromboplastin. Then add 0.1 ml of $CaCl_2$. Start the stop watch and note the time when clot forms. Test should be done with control plasma.

Normal range: 0.8–1.2 seconds

PT international normalized ratio (PT-INR): (PT test/PT control) ISI

ISI: International Sensitivity Index for any tissue factor they manufacture (WHO-primary thromboplastin). The ISI is usually between 1.0 and 2.0.

Normal range of PT-INR: 0.9–1.3.

ACTIVATED PARTIAL THROMBOPLASTIN TIME (APTT)

Clinical Significance

- Intrinsic coagulation pathway
- Deficiency of factor VIII, IX, XI, XII
- Deficiency of common pathway

Principle

Partial thromboplastin is incubated with Kaolin and clotting time is noted after adding calcium.

Procedure

To 0.1 ml of brain extract add 0.1 ml of Kaolin, add 0.2 ml of patients plasma. Incubate at 37°C for 1 minute and add 0.1 ml $CaCl_2$. Start the stopwatch and note the time for clot formation. Test should be done with control plasma.

Normal range: 30 to 40 seconds.

THROMBIN TIME (TT)

Clinical Significance

Fibrinogen converted to fibrin.

Abnormal Values

- Decreased level of fibrinogen
- Qualitative abnormality of fibrinogen
- Presence of heparin/heparin-like substance.

Procedure

To 0.2 ml of plasma add 0.1 ml of thrombin and $CaCl_2$. Start the stopwatch and note time of clot formation. Test should be done with control plasma.

Normal range: 15 to 20 seconds.

Exercise

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LE Cell Phenomenon

Lupus erythematosus (LE) cell phenomenon detects anti-nuclear protein antibody which occurs in serum of SLE, Sjögren's syndrome, rheumatoid arthritis, thyroiditis, myasthenia gravis, cirrhosis and discoid lupus erythematosus (Fig. 1.42).

LE factor has the property of causing *in vitro* lysis of nuclei of neutrophil and subsequent phagocytosis of lysed nuclei by other neutrophil.

LE cell has a typical amorphous pale purple body, surrounded by the nucleus of a polymorph. Rarely monocyte and eosinophil engulf LE body. The LE body may be found extracellularly also.

LE cell has to be differentiated from Tart cell (Fig. 1.43) where in monocyte or rarely a neutrophil does engulf another cell or the nucleus of another cell (most often nucleus of lymphocyte). Here the chromatin pattern of engulfed nucleus is retained without any change.

Procedure to Demonstrate LE Cell Phenomenon

1. ***Blood clot method of Zimmer and Hargraves:*** In this method blood is allowed to clot and is incubated. With two wooden applicator sticks, the clot is mashed into its own serum. The mixture is centrifuged and smears prepared with buffy coat.
2. ***Blood clot method of Magath and Winkle:*** The clot is squeezed (passed forcefully) through a wire sieve or strainer and then

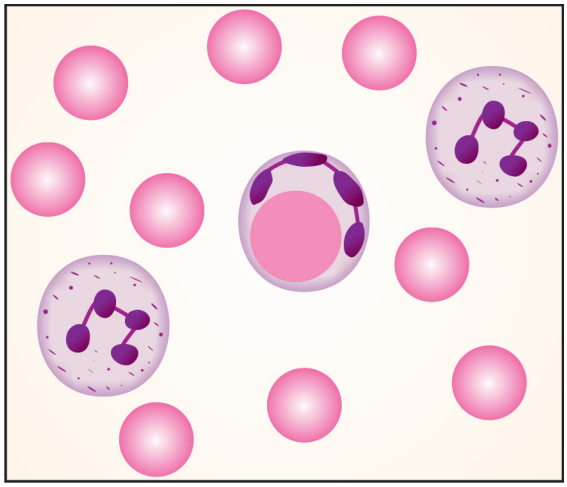

Fig. 1.42: LE cell

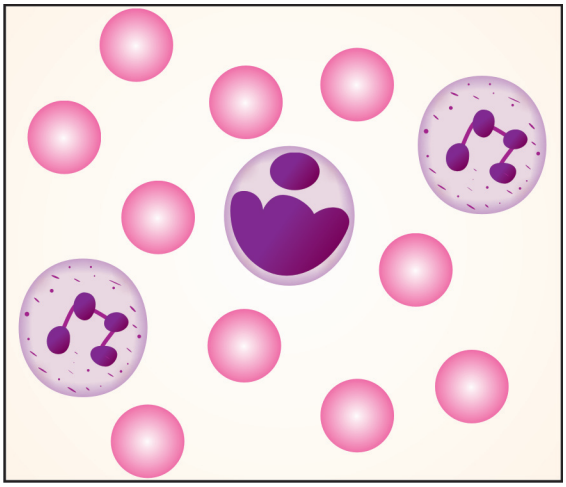

Fig. 1.43: Tart cell

the smears are prepared in a similar way as in the above procedure.

3. ***Zinkham and Conley method:*** The heparinised blood is traumatised by glass beads for 30 minutes and smears are prepared in a similar way. For demonstration of LE cell phenomenon, trauma to the leukocytes is necessary.

LE factor does not appear to act on healthy leukocytes. To achieve certain degree of trauma to the leukocytes, different methods are employed before making the smears like shaking with glass beads, mashing the blood clot with wooden applicator, passing forcefully through a wire sieve or strainer or by squeezing.

Examine the films under low power (20X) magnification and confirm under oil immersion. Slide should be examined at least for 10 minutes before a negative report is arrived at.

Exercise

21

Romanowsky Stains, Buffer, Instruments and Cleaning of the Glassware

ROMANOWSKY STAINS

Romanowsky dyes consist of a mixture of basic dye that is methylene blue and an acidic dye eosin. Methylene blue stains the acidic cell components (nucleus and cytoplasmic RNA). Eosin is red coloured, and it stains the basic components such as haemoglobin.

A number of Romanowsky stains are available which are used singly or in combination.

- Wright's stain
- Leishman stain
- Giemsa stain
- May-Grünwald Giemsa stain
- Jenner-Giemsa stain.

Wright's Stain

Actually, it is a polychromatic stain as it produces a variety of colours. It is a methyl alcoholic solution of an acidic (eosin) and a basic (methylene blue) dye. Wright's stain powder is also available commercially.

Wright's power is obtained after drying a solution containing:

Methylene blue	1 g
Sodium carbonate 0.5%	100 ml
Eosin w/s (yellowish) 0.1% aqueous solution	500 ml
Methyl alcohol (absolute)	60 ml

Preparation of Wright's Stain

Wright stain containing about 2.5 g of Wright's powder in 1 L of chemically pure absolute methyl alcohol (acetone free) is prepared. The powder is ground in a mortar by adding a few ml of methyl alcohol till 1 L have been added. This step requires 20–30 minutes. The stain is left standing for a day or two and then it will be ready for use. Few laboratories add about 30 ml of glycerin to the Wright's powder, mix it well in a mortar. Incubate this for 24 hours at 37°C and then mix with 1 L of methanol.

The dye is sensitive to water and detergents. So the bottle should be tightly stoppered to prevent entry of water vapour. Exposure to acid or alkali should be totally avoided.

Structures which are stained with basic dyes are basophilic. Structures that take up acidic dye are called acidophilic, whereas structures which are stained with both are called, neutrophilic.

Leishman Stain

0.2 g of powdered dye is taken in a conical flask of 200–250 ml capacity. 100 ml methanol is added and then warmed to 50°C for 15 minutes with occasional shaking. The solution is ready for use.

Note: The alkaline pH accentuates the methylene blue component and *vice versa* is also true. pH of 7.2 is recommended for malarial parasites in order to demonstrate Schuffner's dots.

Giemsa Stain

It is the best stain for identifying blood parasites and other protozoa.

Composition

Azur II eosin	3.0 g
Azur II	0.8 g
Glycerin (Merck CP)	250 ml
Methyl alcohol	250 ml

BUFFER

Buffer maintains the pH of the stain solution. For staining the peripheral smear, buffer of pH of 6.4–6.7 is preferred.

Buffer for Wright's stain (pH 6.4): The contents of the buffer are as follows:

Primary (monobasic) potassium phosphate (KH_2PO_4), anhydrous 6.63 g; secondary dibasic sodium phosphate Na_2HPO_4, anhydrous 2.56 g and distilled water up to 1 litre are added.

For pH of 6.7, about 5.13 g of potassium salt and 4.12 g of sodium salt are used. In case of the films stained with Wright's stain, the RBCs should stain pink and not lemon yellow or red and the nuclei of leukocytes should stain purple.

The distilled water placed in a glass bottle for at least 24 hours (aged distilled water) has the pH of 6.4–6.8 can also be used.

Different Methods for Cleaning of the Glasswares

1. Wash the slides with soap and water and then with abundant clean hot water followed by distilled water. Dry them and polish with lint free cloth. Only edges should touched and these slides can be stored in a slide box for use when required.
2. The slides, cover glasses and other glasswares can be cleaned with a mixture of dichromate and H_2SO_4. They can be dropped into the above solution and left in it for 4 to 24 hours. Later the solution is poured off and the slides are washed with multiple changes of tap water. Complete removal of acid is assessed using litmus paper. The litmus paper should show neutral pH and then the slides are washed with distilled water, dried and stored.
3. Dirty slides are put into some detergent solution; heated to 60°C for 20 minutes, washed in hot running tap water and dried with clean linen cloth.
4. The glass slides are washed in running tap water, boiled in some detergent solution, rinsed in acid and washed in hot running tap water.

Exercise

22

Automation in Haematology

Automation is the process where haematology tests are performed by humans by computerised methods. Until recently haematological tests were performed by manual methods. Both the manual and automated laboratory techniques have their own advantages and disadvantages. It is very unlikely that one method will completely replace the other.

AUTOMATED HAEMATOLOGY ANALYSER
(Figs 1.44 and 1.45)

It is of two types.

A. Semi-automated—few parameters are only performed. Few steps are performed manually by technologists requiring more time.

B. Fully automated—measures multiple parameters in less time.

Principles of Working

Automated haematology analysers work on different principles or combination of different principles.

- Electrical impedance
- Light absorption
- Light scatter
- Electrical conductivity
- Fluorescence.

Electrical Impedance

As this was first developed by Coulter electronics it is also known as Coulter principle. In an isotonic solution two electrodes are separated by a glass tube having a small aperture. Cell passes through the aperture after applying vacuum, the flow of current is impeded and a voltage pulse is generated. The height of pulse is proportional to the cell volume and the width corresponds to time taken to traverse the aperture.

Anticoagulated blood is aspirated into the system which is divided into two portions and mixed with diluents. One dilution passed through RBC aperture (for RBC and platelet counting) and the other through WBC aperture (for WBC and Hb estimation). Haemoglobin is estimated by light transmission at 535 nm (Fig. 1.45). RBCs are counted between 36 and 360 fl, platelets between 2 and 20 fl, 35 and 90 fl are lymphocytes, 90 and 160 fl are mononuclear cells, and neutrophils between 160 and 450 fl.

Light Absorption

Haemoglobin is converted to cyanmethaemoglobin or other compound and measured by absorption spectrophotometry. Leukocytes are classified by peroxidase cytochemistry. The peroxidase activity is detected by principle of absorbance.

Light Scatter

Cells flow or move in a single line. Laser device is focussed on the cell flow, the laser light beam strikes the cell and is scattered in various directions. There are two cell detectors. The forward scatter light is detected

Fig. 1.44: Cell counter—3 part differential count

Fig. 1.45: Cell counter—5 part differential count

by one detector and it is proportional to cell size. The second detector captures side scatter which assess the nucleus and granules in the cytoplasm. The simultaneous scatter in both directions helps to differentiate the granulocytes, lymphocytes and monocytes.

Electrical Conductivity

High frequency current is used to determine and classify leukocytes by their physical and chemical composition.

Fluorescence

RNA (reticulocytes), DNA (NRBCs) and cell surface antigens can be measured by cellular fluorescence.

Parameters Measured by Haematology Analysers

- Routine analysers
- Upgraded analysers
- RBC count
- Reticulocyte count
- WBC count (TC)
- Red cell distribution width
- WBC differential count (DC)
- Reticulocyte haemoglobin content
- Platelet count
- Mean platelet volume (MPV)
- Mean cell volume (MCV)
- Platelet distribution width (PDW)
- Mean cell haemoglobin (MCH)
- Reticulated platelets.

Mean Cell Haemoglobin Concentration (MCHC)

The haematology analysers produce the RBC, WBC and platelet results in the form of histograms, scattergrams.

The haematology analysers could be three part differentials where WBC differential is reported as granulocytes, lymphocytes and monocytes and it works on the principle of electrical impedance or 5 part differential where neutrophils, basophils, eosinophils, lymphocytes and monocytes are measured by using combination of different principles.

Advantages

- Accurate and precision in tests
- Can perform multiple tests on a single platform

- Significant reduction in manpower
- Speed and efficient handling of large number of samples
- Accurate determination of red cell indices in automated haematology analyser.

Disadvantages

- Erroneous results due to interfering factors
- Expensive machine and reagents
- RBC morphology (shape and size) at times not recognised
- Hence, flags develop which have to be confirmed by manual examination
- Machine maintenance.

Flagging

These are the signals generated by the analyser when abnormal results are detected. It reduces the false-positive and false-negative results requiring manual review of the blood smear examination.

Platelet Function Analyser (PFA-100)

It is a screening test for platelet adhesion and aggregation. Anticoagulated blood is passed through small membranes coated with either collagen and epinephrine or collagen and ADP. The platelets adhere to the membranes and occlude the aperture at the centre of the membrane. Normal closure time is 1–3 minutes.

Normal PFA with collagen/epinephrine: No significant platelet function defect.

Prolonged collagen/epinephrine and normal collagen/ADP—aspirin induced platelet defect.

Prolonged collagen/epinephrine and collagen/ADP—congenital or acquired platelet function defects.

Haemoglobin Estimation (Fig. 1.46)

The HaemoCue haemoglobin photometer has been widely used for as a point-of-care device for haemoglobin estimation in mobile blood donations and critical care areas in health facilities. However, it is not recommend in general practice.

Fig. 1.46: HaemoCue for haemoglobin estimation

Automation to Determine Hb Variants

Inherited haemoglobin disorders, haemoglobinopathies and thalassaemias, largely originated in the tropics, but now are common worldwide due to migration. At least 5.2% of the world population (and more than 7% of pregnant women) carry a haemoglobin variant. It is also estimated that around 1.1% of couples worldwide are at risk for having children with a haemoglobin disorder, and 2.7 per 1,000 conceptions are affected.

The haemoglobinopathies, or Hb variants, are attributable to amino acid substitution(s) in either globin chain.

DETECTING Hb DISORDERS, WITH AN EMPHASIS ON CAPILLARY SEPARATION

Automated Separation Methods

Most large laboratories currently use automatic high-throughput methods, such as high-performance liquid chromatography (HPLC) and/or capillary electrophoresis (CE). With virtually 100% sensitivity, these methods easily identify elevated HbA2 in β-thalassaemia and common Hb variants.

High pressure liquid chromatography: On HPLC, haemoglobin samples are injected into a resin column and separated based on charge. Haemoglobin variants elute from the column

and are detected at 415 nm, then at 690 nm to correct the baseline. The haemoglobin retention time (from injection until the maximum point of each peak) is calculated and plotted on a chromatogram. Glycosylated fractions and other post-translational adducts separate from the main peaks, making the chromatogram somewhat challenging to interpret.

HPLC has been implemented in the clinical laboratory for evaluating haemoglobin abnormalities. HPLC systems provide automation, allow for precise quantitation of HbA2 (with some exceptions), and are effective at detecting common and rare variants. Some rare variants could be missed on HPLC, where they would be detected on CE or other technique, but the opposite is also true.

Capillary Electrophoresis (CE) Separation Technology

Capillary electrophoresis separates haemoglobin variants by electro-osmotic flow and electrophoretic mobility in alkaline buffer (pH 9.4). Multiple samples (two to eight, depending on the instrument used) undergo high-resolution separation concurrently in silica glass capillaries, taking approximately eight minutes to complete the analysis. For Hb variant detection, UV at 415 nm wavelength is used. The detection methodology is similar to one used in HPLC systems. As a result, the methodology is sometimes considered a "hybrid" type of separation technique between classical zone electrophoresis and liquid chromatography.

An electrophoregram consists of 300 consecutive readings and is divided into 15 zones that are either numbered (i.e. Z1) or named according to the common variants (e.g. Z(S) for the zone where HbS migrates). Haemoglobin variants are displayed as peaks, and the zones where the variants belong are automatically marked by the system. All normal haemoglobins (HbA, HbA2 and HbF) are automatically identified. An on-board drop-down library assists with the interpretation of the results.

Significant changes have been made in the latest generation CE systems in order to provide complete automation and improve workflow in the laboratory, while maintaining separation profiles and results identical to those from previous generation instruments. Such systems run two whole-blood programmes.

Diabetes Mellitus and Haemoglobin Variants

Correct interpretation of HbA1c measurements depends on normal erythrocyte lifespan. In individuals with sickle cell, HbC, or HbD disease it is recommended that tests other than HbA1c be used for the determination of glycemic control (e.g. glycated serum albumin), since the lifespan of red blood cells is altered. If heterozygous carriers show normal erythrocyte survival, HbA1c can be used as long as the haemoglobin variant does not interfere with the assay method, nor with glucose binding to haemoglobin. There is some evidence to suggest that Hb variants may affect RBC life-span even in Hb trait patients who are asymptomatic. Thalassaemia also can affect lifespan of RBC, resulting in a markedly low HbA1c value.

Automation in Reticulocyte Count

Using an seven part automated haematology analyser, the automated absolute reticulocyte count, the reticulocyte index, or reticulocyte production index (RPI), immature reticulocyte fraction (IRF) can be obtained.

Measurement of Reticulocyte-specific Haemoglobin Content

The automated haematology analyser can also report a measurement of reticulocyte-specific haemoglobin content as mean reticulocyte haemoglobin content (CHr) or reticulocyte haemoglobin equivalent (Ret-He), depending on the type of instrument used. CHr and Ret-He, two comparable but not identical

parameters, give a snapshot of the functional iron available for incorporation into haemoglobin within RBCs over the previous 3–4 days. A decreased value generally reflects reduced cellular haemoglobin content and is reliable in identifying functional iron deficiency. Furthermore, this parameter is the strongest predictor of iron deficiency anaemia in children.

Indications

The reticulocyte count is not usually a part of a standard CBC count, but is ordered and used along with CBC, as CBC with reticulocyte count, to guide anaemia workup or response to treatment. Depending on the type of automated haematology analyser used in the laboratory, reticulocyte-specific haemoglobin content and IRF may be reported along with reticulocyte count to provide additional valuable information. These parameters are useful in the following situations:

- Anaemia workup (peripheral destruction vs failure of production)
- Response to therapy (iron, vitamin B_{12}, folic acid supplementation)
- Bone marrow recovery after bone marrow transplantation or intensive chemotherapy.

Flow Cytometry

It is a procedure which measures multiple cellular and fluorescent properties of cells. The instrument is called flow cytometer.

Principle: The cells suspended pass through a single flow suspension through a laser beam. Cells of 0.2 to 150 μm are analysed by flow cytometric analysis.

It has three main components

1. *Fluidics:* It transports the cells in a stream to the laser beam for interrogation.
2. *Optics:* It consists of lasers-argon-ion laser for illumination of cells.
3. *Electronics:* The optical signals are converted to corresponding electronic signals by photodetectors.

Hence, the flow cytometer provides following information of cell.

- Cell size
- Granularity or internal complexity
- Relative fluorescence intensity.

Applications in Haematology

1. Diagnosis, prognosis and to assess minimal residual disease in leukaemias and lymphomas.
2. Diagnosis and prognosis of leukaemias, MDS, plasma cell neoplasms, mast cell neoplasms.
3. Enumeration of CD34 positive cells.
4. It is used in haemopoietic stem cell therapy, targeted therapy.
5. Protein profiling.
6. Measurement of drug uptake and multi-drug resistance proteins.
7. Identification of leukaemic stem cells/side population cells.
8. Newer application in RBC disorders—PNH, antibody detection in AIHA, feto-maternal haemorrhage, fetal RBC measurement in haemoglobinopathies and myelodysplasia, reticulocyte analysis, immature reticulocyte fraction, alloimmunisation detection, transfusion related immunologic reactions, erythrocyte phenotyping, rheology, bone marrow engraftment and regeneration evaluation, sickle cell and thalassaemia monitoring, parasite infection detection, congenital chimerism and mosaicism detection.
9. Helpful in diagnosis of primary immunodeficiency disorders.

1. How do you differentiate coagulation from platelet or vascular disorders?

Finding	*Coagulation disorder*	*Platelet or vascular disorders*
1. Haematoma	Characteristic	Rare
2. Ecchymosis	Common, large and solitary	Common, small and multiple
3. Petechiae	Rare	Characteristic
4. Haemarthrosis	Characteristic	Rare
5. Delayed bleeding	Common	Rare
6. Bleeding from superficial cuts and scratches	Minimal	Persistent
7. Sex	Males in the family	Relatively common in females
8. Family history	Present	Rarely present

2. What are the causes of megaloblastic and non-megaloblastic anaemia?

Megaloblastc anaemia: Caused by B_{12} and folic acid deficiency.

Non-megaloblastic anaemia: Causes are listed below:

a. Myelodysplastic syndrome (MDS)
b. Liver dysfunction
c. Alcoholism
d. Hypothyroidism
e. Drugs
f. Rare RBC disorders: Congenital dyserythropoietic anaemia, Diamong–Blackfan syndrome

DISSEMINATED INTRAVASCULAR COAGULATION

Disseminated intravascular coagulation (DIC) is the systemic uncontrolled activation of coagulation leading to widespread deposition of fibrin, with formation of microvascular thrombosis. During this coagulation process there is:

- Coagulation factor deficiency
- Decreased platelet count
- Massive bleeding

Patients with DIC can have: renal, hepatic, respiratory failure as well as central nervous system and cutaneous sequelae. Following are the clinical conditions associated with DIC.

- Sepsis
- Solid tumours (lung, breast, stomach, prostate, pancreas, ovarian, biliary tract)
- Haematologic malignancies (acute promyelocytic leukemia)
- Obstetrical complications (HELLP syndrome, amniotic fluid embolism, eclampsia)
- Acute pancreatitis
- Trauma
- Severe transfusion reactions (acute haemolytic transfusion reactions)
- Snake venoms
- Heat stroke and hyperthermia

Mechanism

There are many causes for DIC. These trigger the activation of coagulation cascade intravascularly. There is development of fibrin webs and the activation and aggregation of platelets inside the capillaries lead to microvascular thrombosis leading to tissue ischaemia and multi-organ failure.

The concentration of circulating coagulation factors decrease with activation of coagulation. Since intravascular thrombosis consumes coagulation factors, this condition is referred to as a consumptive coagulopathy. The risk of bleeding rises as coagulation factor concentration reduces and platelets are reduced in count as a result of aggregation in the circulation. Thus, thrombosis and bleeding occur simultaneously with DIC.

Signs and Symptoms

These depend upon on the cause and whether condition is acute or chronic. Acute DIC develops quickly and is a serious condition. Chronic DIC develops slowly and lasts longer. With acute DIC, there is:

- *Spontaneous bleeding*: Spontaneous bleeding from different sites including the ears, nose, digestive, genitourinary, respiratory systems, wounds or sites of venepuncture or cannulation. The presence of bleeding from three unrelated locations strongly suggests DIC. Bruising that is widespread or sudden and does not have a trauma history.
- *Thrombosis:* Digital ischaemia/gangrene, renal cortical necrosis, haemorrhagic adrenal infarction.

Laboratory Investigations

Important laboratory investigations include:

1. Thrombocytopenia in DIC due to excessive consumption
2. Coagulation screen: PT and APTT prolonged
3. Fibrinogen: Decreased as fibrinogen is converted to fibrin in intravascular thrombosis
4. D-dimer/fibrin degradation products: Raised due to degradation of fibrin

International Society of Thrombosis and Haemostasis Scoring System

The International Society of Thrombosis and Haemostasis (ISTH) has produced a scoring system. This scoring system utilises the platelet count (>100/µl, 50–100/µl, <50/µl), D-dimer value (not increased/moderate/high), prothrombin time(prolongation ≤3 sec/3–6 sec/>6 sec) and fibrinogen levels (>1 g/L and <1g/L) to assess the likelihood that a patient has DIC. Zero, 1 and 2 points are given. A score of ≥5 indicates overt DIC.

Differential Diagnosis

- Acute hepatic failure
- Vitamin K deficiency
- HELLP syndrome
- Idiopathic purpura fulminans

Complications

1. Multi-organ failure
2. Haemorrhage
3. Cardiac tamponade
4. Haemothorax
5. Intracranial haemorrhage
6. Gangrene and loss of digits

SICKLE CELL ANAEMIA

Sickle cell anaemias are hereditary disorders in which red cells contain Hb-S. Haemoglobin-S differs from Hb-A in substitution of valine for glutamic acid in 6th position from the N-terminal end of beta chains.

Geographical Distribution and Prevalence

HbS is prevalent in Africa, Mediterranean countries, and India. Black population of USA and Latin America have also found this gene. In India, it is found in Orissa, Andra Pradesh and Madhya Pradesh.

Hb-S begins to sickle at oxygen tension of 50–60 mm of Hg. Hb-S cells are sensitive to pH. The decrease in pH from 7.4 to 7.2 results in:

1. Failure of sodium and potassium pump. Sodium enters and potassium leaves the cells.
2. There is increased calcium ion concentration.
3. RBCs get sickled.
4. Sickled RBCs abnormally adhere to endothelial cells
5. In deoxygenated condition, the viscosity of plasma proteins increases.

Clinical Features

1. Dactylitis and pain
2. Acute chest syndrome
3. Priapism
4. CNS events

5. Aplastic crisis—due to parvovirus infection
6. Haemolytic crisis
7. Veno-occlusive crisis
8. Sequestration crisis
9. There is increased susceptibility to infections due to hypofunctioning of spleen.
10. Infections:
    i. *S. pneumonia* until 5 years of age
    ii. *E. coli* in older children and adults
    iii. Osteomyelitis: *Staphylococcus* and *Salmonella*
11. Chronic organ damage
12. Leg ulcers
13. Common age of presentation is 3 months to 1year of age
14. The height and weight is reduced. There is delayed puberty
15. Spleen gradually enlarges. After 5–6 years, there is gradual reduction size of spleen
16. Multiple infarcts and fibrosis of spleen lead to decreased size (autosplenectomy).
17. Hand and foot syndrome: Hands and feet are painful and swollen. There is destruction of phalanges, metacarpal and metatarsal bones
18. Hepatomegaly and gallstones
19. Cardiomegaly with systolic murmurs

Laboratory Investigations

1. Haemoglobin: Low (5–10 g/dl)
2. HbF: Positive
3. TC: Increased (20–30 × 10^9/L)
4. Platelets: Normal or increased
5. Peripheral smear: Usually normocytic normochromic anaemia, with normal MCV and MCHC. With deoxygenated states may have sickled cells may be present
6. Occasional target cells and Howell–Jolly bodies are present
7. Reticulocytes may range 10–20%
8. ESR: Low as with sickled cells Rouleaux formation does not takes place
9. Serum folate and red cell folate levels are low
10. Seum haptoglobin and haemopexin: Low
11. Serum iron and ferritin and transferritin saturation: Increased
12. Free haemoglobin and methaemalbumin: May be present
13. Sickle test: Positive
14. Haemoglobin solubility tests: Positive
15. Family studies
16. Hb electrophoresis for HbS and HbA

Solubility test: Sickle cell Hb is insoluble in deoxygenated state in a high molarity phosphate buffer. Crystals formed refract light and solution to appear turbid. Positive test–HbS. The test does not differentiate between homozygous and heterozygous states (Fig. 1.47).

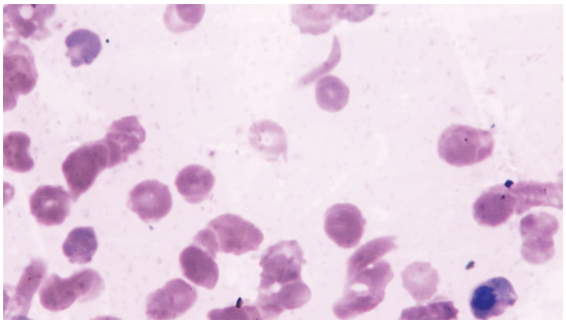

Fig. 1.47: Shows pripheral smear in sickle cell anaemia. Note: Sickled RBCs, nucleated RBC and increased polychromatophilic cells

Autoimmune Haemolytic Anaemia

These are haemolytic anaemias due to development of antibodies directed against antigens on the surface of patients own cells. hese antibodies are usually IgG type and less commonly IgM or IgA and some bind complement.

Autoimmune haemolytic anaemia can be due to following causes:

1. Idiopathic

a. Warm antibodies: IgG and C3 or only IgG, DCT positive
b. Cold antibodies: Paroxysmal cold haemoglobunuria (PCH), cold haemagglutination disease(CHAD)

2. **Secondary drugs:** M-dopa, mefenamic acid, L-dopa, procainamide:
   These act by two mechanisms:
   i. Drug adsorption on the RBC surface and antibodies are formed against this
   ii. Drug combines with proteins and antibodies formed against this complex on the surface activates complement

Red cells coated with IgG and C3 or only with IgG are destroyed by the spleen (Extravascular destruction.

CHAD is associated with malignant lymphoma, CLL, SLE, Waldenstorms macroglobulinaemia, infectious mononucleosis, and *Mycoplasma*.

COMPATIBILITY TESTING

Objectives

- What is compatibility testing
- What is the need
- Blood grouping of recipient
- Explain compatibility testing procedure

What is compatibility testing?

This is procedure is done to determine compatibility between donor and recipients' blood.

***Purpose*:**

a. Accurate blood grouping and Rh typing of recipient and selection of donor with same blood group and
b. Should have no haemolysis or any agglutination, if they are mixed together.

What is the need for this testing?

The main blood group systems are ABO and Rh blood groups. This mainly depends upon the presence of antigen on the surface of RBCs. The ABO system obeys Landsteiner laws, i.e. if particular antigen is present on the wall of the RBC its corresponding antibody is missing in the serum. Vice-versa is also true. If antibody is present the serum, its corresponding antigen is not present on the surface of RBCs.

In Rh system only antigen is present. Its antibody is not present normally.

Blood transfusion given with some indication to benefit the patient. The benefits and disadvantages to be overweighed before transfusing.

Thus compatibility testing is done to know whether the blood cells of the donor should survive in the recipient's blood and do good to him.

Blood grouping of the recipient

Do ABO grouping and Rh typing by forward grouping and reverse grouping.

In forward ABO grouping, reaction of patient red blood cells tested with reagent anti-A and anti-B and anti-AB anti-sera by slide method confirmed by tube technique/ matrix gel card method. In reverse grouping reaction of patient *serum* with known group A and group B cells. Similarly, Rh typing is also done forward and reverse grouping.

Compatibility Testing Procedure

This can be:

- Major crossmatching
- Minor crossmatching

Major crossmatch

Donor's red cells are mixed with recipient's serum.

This procedure determines compatibility between red cells of donor and serum (plasma) of the recipient.

Minor crossmatch

Donor's serum is mixed with recipient's red cells.

This procedure determines compatibility between serum (plasma) of donor and red cells of the recipient. However this is not used routinely.

Immediate spin cross match is preferred when:

1. No clinically significant antibodies are detected in the antibody screen
2. No record of previous detection of clinically significant unexpected antibodies.

This procedure requires patient's serum to be mixed with saline suspended red cells (2–4% conc.) of donor at room temperature.

Major crossmatch: In a test tube, add two drops of patient's serum and one drop of donor cells.

Minor crossmatch: In a test tube add two drops of donor serum and one drop of patient's cells.

The tubes run immediately. Centrifuge at 1000 rpm for 1minute.

Result: Compatible when no agglutination or haemolysis seen:

If clinically significant antibodies are present with Coombs' test/antibody screen: Major crossmatch is done by:

Incubation at 37°C followed by indirect antiglobulin test.

Result: Compatible when no agglutination or haemolysis.

BLOOD COMPONENTS AND THEIR CLINICAL USE

The blood components are necessary for the following reasons:

1. From whole blood various components can be prepared. Whole blood storage conditions are not optimal for all functional components of blood. For example after 24 hours of storage at 1 to 6°C. whole blood has few viable platelets and granulocytes. Stable coagulation factors are well maintained during storage, but heat labile factors like factor V and VIII decrease with time and may not be adequate to correct specific deficiencies in patients. The separation of various components, allow storage of each component at the temperature and storage conditions required for *in vitro* survival.
2. Component preparation allows transfusion of specific portion of blood product that the patient requires.
3. Transfusion of required components avoids use of unnecessary whole blood transfusion which could be contraindicated in some conditions. For example, risk of hypervolaemia in an elderly anaemic patient with congestive cardiac failure may not tolerate the transfusion of two units of whole blood.
4. Also, whole blood exposes to unnecessary antibodies. It also has white blood cells and platelets which can carry HLA antigens and can stimulate the patient's immune system.

Indications

Indications for different components are given below.

Red cells transfusion

1. Patients with symptomatic anaemia
2. Newborn exchange transfusion.
3. Major surgeries
4. Haemorrhage
5. Leukaemia
6. Intrapartum haemorrhage
7. Thalassaemia cases

Transfusion of platelets

1. In prophylaxis of haemorrhage: Platelet count of $<10 \times 10^9$/L, coagulation disorders, petechiae, ecchymosis
2. Prior to surgical or invasive procedures, with platelet count of $<50 \times 10^9$/L
3. In case of microvascular bleeding without thrombocytopenia, when impairment of platelet function is confirmed by laboratory tests. Platelets are transfused if this impairment cannot be treated by other ways (e.g. in case of congenital platelet disorders).
4. Performing epidural anaesthesia or analgesia, with platelet count of $<100 \times 10^9$/L.
5. In case of normal vaginal delivery, thrombocytopenia of $<50 \times 10^9$/L is safe without need for prophylactic platelet transfusion.

Transfusion of fresh frozen plasma (FFP)

1. Coagulopathy and bleeding of different origin
2. Bleeding in case of disseminated intravascular coagulation (DIC)
3. Thrombotic thrombocytopenic purpura

4. Congenital or acquired deficiency of different coagulation factors, when there is no possibility to get a certain factor concentrates (e.g. V or XI)
5. Deficiency of specific plasma proteins (e.g. antithrombin III)
6. Bleeding due to warfarin therapy.

Transfusion of cryoprecipitate

1. Factor VIII deficiency
2. Haemorrhage due to hypofibrinogenaemia or dysfibrinogenaemia
3. DIC syndrome
4. von Willebrand's disease
5. Haemorrhage due to factor XIII deficiency.

Classify transfusion reactions. Explain in brief.

Classification of Transfusion Reactions

Transfusion reactions can be classified as below:

Acute transfusion reactions: immunological

- Febrile non-haemolytic transfusion reaction (FNHTR)
- Allergic reactions
- Anaphylactic and anaphylactoid reactions
- Acute haemolytic transfusion reactions (AHTRS)
- Transfusion related acute lung injury (TRALI)

Acute transfusion reactions: non-immunological

- Bacterial contamination
- Transfusion-associated circulatory overload (TACO)
- Physical and chemical haemolysis
- Metabolic derangements

Delayed transfusion reactions: immunological

- Delayed haemolytic transfusion reactions (DHTRS)
- Transfusion-associated graft-versus-host disease (TA-GVHD)
- Post-transfusion purpura

Delayed transfusion reactions: non-immunological

- Iron overload
- Post-transfusion haemosiderosis
- Transfusion–transmitted diseases

Febrile Nonhaemolytic Transfusion Reaction (FNHTR)

This is a frequent kind of reaction, occurs in 1:200 cases. 1°C temperature rise associated with transfusion of blood when there is no medical explanation for fever other than blood transfusion. Patient has immunologic sensitization to donor WBCs, platelets or plasma proteins. Common sources are prior transfusions, previous pregnancies, previous transplants. HLA class I antigens or leukocyte antigens on the WBCs of the donor are responsible for this reaction.

Following preventive measures are taken:

a. Antipyretics are used to treat fever or are given prior to blood transfusion as a preventive measure.
b. Discontinue blood transfusion if the patient has severe reaction.
c. Pre-storage leukocyte reduced units of red blood cell or platelet packs may also be given.

Allergic Reactions

Patient is sensitized (usually IgE antibody) to foreign plasma antigens. Commonly caused by transfusion of plasma containing blood components, e.g. FFP, cryoprecipitate, platelet concentrates. Allergen forms antigen antibody complex. Attach to mast cells release leukotrienes /histamine causing allergic reaction. Pre-medicate patient with antihistamines. If signs/symptoms are mild and/or transient, restart transfusion after treatment. Do *not* restart transfusion if pulmonary symptoms/signs and fever present. Use plasma deficient blood components. Prophylactically treat with antihistamines.

Anaphylactic and Anaphylactoid Reactions

These reactions can range from mild urticaria to severe shock and death although rare. These occur at the rate of about 1 per 150,000 patients. Pre-formed anti-IgA antibodies are present in the recipient's blood with IgA deficiency which reacts with IgA present in

the donor plasma. If reaction occurs, we use following medication: epinephrine (vasoconstrictors and bronchodilators) and corticosteroids. Use washed RBCs and blood components and transfuse IgA deficient blood.

Acute Haemolytic Transfusion Reactions (AHTRs)

Most common cause for AHTR is ABO incompatibility (clerical error). Incidence: 1:25,000. As little as 10–15 ml can trigger a reaction. Occurs within 24 hours. *Causes*: Transfusion of incompatible donor RBCs into patient. Usually an ABO incompatibility, most commonly antibodies of A,B or AB. Red cell destruction due to complement activation by IgM antibodies.

Steps to be taken when a transfusion reaction occurs due to AHTR

- Stop the transfusion immediately
- An intravenous line with normal saline should be maintained
- Obtain vital signs
- Begin O_2 if pulmonary symptoms are prominent
- Obtain a new blood sample for repeat ABO compatibility test and for evidence of haemolysis
- Obtain a urine sample, if the patient can void
- Obtain a chest X-ray if pulmonary symptoms are prominent
- Physician is notified
- Bedside clerical checks of all forms, labels and patient identification for correctness of the unit and the intended recipient are required
- The unit and all tubing should be returned to the blood bank, along with post-infusion blood and urine samples
- Finally, the reaction should be documented in the patient's chart
- Once these initial measures have been implemented, the investigation of the reaction by the transfusion service can proceed.

This type of reaction is a most dangerous immunologic complication of red cell unit transfusion.

- It carries high risk of morbidity or mortality
- Morbidity is because of renal failure and DIC
- Mortality: Occurs in 1 per 100,000 transfusions

Prevention

Preventing or detecting errors in every phase of the transfusion process such as:

- Sample acquisition
- At all steps in laboratory testing
- At the time of issue
- At the time of transfusion
- Good manufacturing practices with written standard operating procedures should be followed
- Perform pre-transfusion compatibility testing
- Ensure that all clinical staff to recognise signs and symptoms of acute haemolytic transfusion reaction.

Laboratory Investigations in AHTRs

On pre-transfusion sample: Reconfirm ABO, Rh and antibody screen tests.

On post-transfusion sample: Following are to be undertaken:

- ABO, Rh, antibody screen
- DCT
- Urine for haemoglobinuria
- Serum bilirubin
- Haemoglobinaemia (>50 mg/dl)
- Blood urea, creatinine
- Urine output
- Coagulation screen
- Identify discrepancies

Transfusion Related Acute Lung Injury (TRALI)

This occurs in 1 in 5,000 transfusions. Patient displays acute respiratory insufficiency with X-ray showing bilateral symmetric pulmonary oedema. Leukocyte antibodies of donor react with patient's leukocytes and *vice versa*.

Activate complements and damage endothelium of lung capillaries.

Acute Transfusion Reactions: Non-immunological

Bacterial contamination. Treat with broad spectrum antibiotics.

Transfusion Associated Circulatory Overload (TACO)

Patients at significant risk are:

- Children
- Elderly patients
- Chronic anaemia
- Cardiac disease
- Thalassemia major, sickle cell disease or congenital haemolytic anaemias

Physical and Chemical Haemolysis

Improper storage: Overheating or freezing.

Improper preparation: Freezing without cryoprotective agent.

Mechanical stress: Cardiopulmonary bypass pump.

Metabolic Derangements

- Citrate toxicity
- Hyperkalaemia
- Hypothermia
- Coagulopathy in massive transfusion
- Air embolism

Delayed Haemolytic Transfusion Reactions (DHTRs)

It may not be recognised for weeks or months after transfusion. This is due to anamnestic reaction mediated by IgG antibodies on the following occasions. Patient previously exposed to RBC antigen and has low antibody titre until exposed again.

- Antibodies to Rh, Kidd, Duffy, and Kell blood groups
- DAT is negative at first, but becomes positive later. These patients must be given antigen negative blood.

Transfusion-associated Graft-versus-Host Disease (TA-GVHD)

Patient at risk are:

- Immuno-compromised patients
- Newborn and geriatric patients
- Patients with bone marrow transplantation
- Patients on chemotherapy
- Patients with radiation treatment
- Patients who receive relatives blood

Caused by donor lymphocytes, which are transfused into an immune-compromised recipient. *Pancytopenia* occurs as a result of the immunologic response. *Prevention:* Irradiation of blood components is safer.

Post-transfusion purpura: thrombocytopenia as a result of anamnestic production of platelet alloantibody.Usually occurs in multiparous women who do not have the antigen.

Iron overload

1 unit of Packed Red Cells has 250 mg of iron.

Iron that can be removed by the body: 1 mg/day.

Thus, with multiple transfusions Iron accumulates in tissues and causes haemosiderosis.

Post-Transfusion Haemosiderosis

- Affected organs: Heart, liver, endocrine glands.
- Occurs in individuals who receive multiple transfusions.
- Excess iron accumulates in macrophages in various tissues (liver, heart, endocrine glands)
- It appears as dark brown granules in the cells.
- May lead to organ failure.
- Therapy: Iron–chelating agents.
- Prevention: Transfuse with young RBCs.

Transfusion Transmitted Diseases (TTDs)

Viral infections: Hepatitis viruses: HBV, HCV; Retroviruses: HIV; Syphilis, Parasitic Infections: Malaria.

Distinguish between direct and indirect hyperbilirubinaemia and list the Causes of Direct and Indirect hyperbilirubinaemia

In the liver, during catabolism of haemoglobin, bilirubin is changed into a form that body can get rid of. This is called conjugated bilirubin or direct bilirubin.

Unconjugated hyperbilirubinemia (Indirect bilirubin) can result from increased production, impaired conjugation, or impaired hepatic uptake of bilirubin, a yellow bile pigment produced from hemoglobin during erythrocyte destruction.

Causes for direct bilirubinaemia include:

a. Alcohol abuse
b. Infectious hepatitis,
c. Drug reactions,
d. Autoimmune disorders.
e. Posthepatic disorders
f. Gallstone
g. Biliary tract infection,
h. Pancreatitis,
i. Malignancies.

Causes of direct (conjugated) hyperbilirubinaemia:

a. Extrahepatic biliary atresia
b. Choledocal cyst
c. Idiopathic neonatal hepatitis
d. Metabolic diseases: Gaucher, Niemann–Pick disease, galctosaemia, tyrosinaemia
e. Alpha 1 anti-trypsin deficiency
f. Sepsis
g. TORCH infections
h. Autoimmune hepatitis
i. Primary biliary hepatitis
j. Primary sclerosing cholangitis.
k. Wilson's disease

Interpretation: Case of Prolonged PT

A 49-year-old woman underwent workup for hip replacement surgery. Hb-12.2 g/dl, TC-9800 cells/mm^3, Platelet count-1.78 lakh/mm^3. PT-20.3 sec (Control-14 sec), INR:1.78, aPTT-30.8 sec. The patient had no history of gastrointestinal or intracranial bleeding, epistaxis, or haemarthrosis. However, she reported a tendency toward easy limb bruising and menorrhagia, which required iron supplementation. She had a negative family history of abnormal bleeding. No pre-analytical errors were identified, and repeat PT-22.8 sec aPTT-30.2 sec.

Interpretation: Prothrombin time is prolonged as Extrinsic and common pathway are affected

The probable causes for its prolongation are: Vitamin K deficiency, Oral anticoagulant therapy, DIC, Inherited deficiency of a coagulation factors like VII, X, V, II and I.

INR is calculated as the ratio of the patients PT/mean PT of a group of normal people and then adjusted for ISI. It is mainly measured for anticoagulant therapy

Interpretation: Case of Prolonged aPTT

A 2-year-old boy was brought to the emergency department by his mother for oozing blood from his mouth following a fall nearly 7 hours ago. His mother gave history of tendency to bleed for prolonged periods from his immunization sites, but there was no history of bruising or haematomas. The patient was on antibiotics for a recent ear infection. There was no known family history of a bleeding disorder. Complete blood count was within normal limits for the age.

PT: 14.2 sec	(Control-14 sec)
APTT: 44.6 sec	(28.0–38.0)
APTT mix: 34.7 sec	(28.0–38.0)
Factor VIII: 0.18U/ml	(0.60–1.50)
Factor IX: 0.92 U/ml	(0.60–1.50)
Thrombin time: 18.3 sec	(16.0–22.0)
VWF AG: 0.88 sec	(0.78–1.53)

Interpretation: Factor deficiency affecting the Intrinsic and common pathway. The probable causes are: Factor VIII, lupus anticoagulant (possible), factor VIII inhibitor (rare at this age).

HAEMATOLOGY CASES

Case 1

A 28-year-old pregnant lady of 16 weeks is seen for easy fatigability for 2 months. She has 3 children in 3 years. She is from a village and has not consulted any doctor.

On examination, conjunctiva is pale, spooning of nails is noted, systolic murmur is present, stool occult blood is negative, haemoglobin is 6.2 g/dl, HCT is 18%, MCV 54 fl, RDW 16.1%, WBC is 6,200/mm^3, DC is normal and platelet count is 2,50,000/mm^3.

Answer the following questions:

1. How do MCV and RDW help you in the diagnostic work-up of anaemia?
2. What are the differential diagnosis for microcytic hypochromic anaemia?
3. What further tests would you like to do in this patient to confirm iron deficiency?

Case 2

A 29-year-old girl was referred for recurrent anaemia. She has easy fatigability and weakness for past two years. She had regular menstruation cycle without increase in blood loss.

Laboratory investigations: Hb ranged between 5 and 6 g/dl, with a low MCV. Hb electrophoresis was normal. Faecal occult blood tests +. She had haemangioma on head. GI endoscopy revealed cavernous haemangioma of the jejunum.

Answer the following questions:

1. Write differential diagnosis.
2. Write diagnosis in the above case.
3. What is the cause of above case?

Case 3

A 15-year-old girl presented with easy fatigability and breathlessness on walking, since few weeks. Patient is vegetarian. Laboratory investigations revealed Hb: 6.2 g/dl, MCV: 110 fl, TC: 1640/µl; platelet count: 1.2 lakh cells/mm^3, S. indirect bilirubin 2.4 mg/dl, lactate dehydrogenase (LDH) is increased, S. cobalamine: 56 ng/L, plasma homocysteine >50 µmol/L. IgA anti-tissue transglutaminase and anti-parietal cell antibodies were negative.

PS examination: Leucopenia and hypersegmented neutrophils present. Bone marrow aspiration study demonstrated megaloblastic erythropoiesis.

Answer the following questions:

1. What is the diagnosis?
2. What are the suggestive findings for the diagnosis?
3. What are other tests required to confirm the diagnosis?

Case 4

A 61-year-old lady, vegan, presented with easy fatigability, loss of weight and appetite. The patient had a history of jaundice 2 months back, for which she had taken medication from a local doctor. There was no history of cough with expectoration, headache, body ache, arthralgia, and rash. On examination systolic murmur+.

***Laboratory investigation*:** Hb 3.2 g/dl, PCV 10.2%, TC 3200 cells/mm^3, platelet count 1.2 lakh cells/mm^3, RBC count 2.6 million cells/mm^3, corrected reticulocyte count 1.6% , RDW 17.5%, MCV112 fl. Peripheral smear showed pancytopenia and hypersegmented neutrophils.

Answer the following questions:

1. What is the diagnosis?
2. How do you explain pancytopenia?
3. What is the role of bone marrow in above diagnosis?

Case 5

A 5-year-old girl, presented with cough, dyspnoea, irritability, and easy fatigability. On physical examination she is anaemic, underbuilt, under-nourished with a short stature, with evident icterus, and yellow tinged fingernails. Head and neck examination showed maxillary expansion depicting the classical Chipmunk facies.

Laboratory investigations:

Haemoglobin: 4.5 g/dl, PCV: 12%

Reticulocyte count 10%, corrected reticulocyte count: 2.6%, MCV <70 fl, RDW: 14.1%, S.iron and s.ferritin—markedly increased.

Transferrin saturation increased, TIBC—normal.

Peripheral smear—microcytic hypochromic anaemia with anisocytosis, poikilocytosis, nucleated red cells+ and target cells+,

Answer the following questions:

1. What is the diagnosis
2. What are the confirmatory tests
3. What are the complications
4. How do you calculate corrected reticulocyte count?
5. How do you differentiate iron deficiency anaemia and thalassaemia minor cases?

Case 6

A 5-year-old male child, from central part of India presented with mild pallor, icterus, and history of on-and-off abdominal pain and joint pains. On examination the child had mild splenomegaly. He has consulted local doctor for fever, anaemia and jaundice. He has had three transfusions till now; last transfusion was 3 months back. There is history of sibling death at 10 years of age due to fever and jaundice.

Laboratory investigation: Hb 7.7 g/dl, RBC count 2.44 million cells/L, MCV 97.1 fl, MCH 31.4 pg, MCHC 32.3 g/dl.

***Peripheral smear*:** Red cell morphology revealed severe aniso-poikilocytosis with macrocytes, target cells, sickle shaped or holly leaf shaped RBCs, and nucleated RBCs. There is leukocytosis.

Answer the following questions:

1. What is the diagnosis?
2. What are the confirmatory tests?
3. What is the genetic defect in sickle cell anaemia?
4. What are the clinical features in sickle cell anaemia?
5. Patients with above condition are prone to which infections?

Case 7

A 22-year-old, male presented with intermittent jaundice of 4 years duration, pain in the left hypochondrium of 6 months duration. He subsequently developed pain in the right hypochondrium, which was intermittent and colicky in nature. He also had exertional breathlessness. No history of fever or blood transfusions in the past. Seven of his family members were affected with similar symptoms. His two maternal uncles had history of prolonged jaundice and cholelithiasis and had undergone cholecystectomy and splenectomy.

Laboratory investigations reveals: Hb 5.8 g/dl, TLC 4000 cells/mm^3, platelets 1.6 lakh cells/mm^3, MCHC 39.3%, MCV 79.3 fl, MCH 31.7 pg. Reticulocyte count 11%, reticulocyte production index was 4.7. Osmotic fragility of incubated blood was markedly increased. Peripheral blood smear showed marked anisocytosis, numerous poikilocytes along with spherocytes.

Answer the following questions:

1. What is the diagnosis?
2. What are the confirmatory tests?
3. What is the cause for abnormal shaped cells?

Case 8

A four-year-old male presents to the emergency department with a history of six days of fever and acute onset of red colored urine. Family history is non-contributory. He has taken medication for fever from a local doctor for a period of 5 days. He has a two-year-old sister who is healthy. Physical examination: scleral icterus+, jaundice+, lab findings: Initial Hb10.4 g/dl; decreased to 6.3 g/dl; 12 hours later, platelets 153,000 cells/mm^3, reticulocyte count 0.4%, TC 11000 cells/mm^3, absolute neutrophil count 7900 cells/L. Coagulation studies are normal. LDH is increased, haptoglobin-low, unconjugated bilirubin is

3.7 mg/dl, Conjugated bilirubin is 1.2 mg/dl, direct antiglobulin (direct Coombs) test: IgG and C3 +.

Urinalysis: Dark, red-brown urine, blood+, 0–4 RBCs/HPF, 0–4 WBCs/HPF.

Answer the following questions:

1. What is the diagnosis?
2. What are the confirmatory tests?
3. What is the cause of this condition?

Case 9

A 45-year-old female with previous history of blood transfusion 6 months back, came with on and off fever since 2 weeks and epistaxis. On examination: Pallor +. USG-fatty changes in the liver.

***Lab findings*:** Hb–8.5 g/dl, WBC–3,920/mm^3, platelets–20,000 cells/mm^3, RBC count–2.8 million cells/ mm^3, HCT–26.5%, MCV–108 fl, MCH–34.02 pg, MCHC–36.5 g/dl, RDW = 13.9% , ESR–-50 mm/hour. Serum iron and vitamin B_{12} within normal limit.

***PS examination*:** All the series cells are reduced, reticulocyte count = 0.2%, no blasts seen.

***Bone marrow aspiration*:** Dry tap, bone marrow biopsy: Hypocellular marrow for the age with increase in fat spaces. No fibrosis present.

Answer the following questions:

1. What is the diagnosis?
2. What are the suggestive findings for the diagnosis?
3. What are the other investigations to be done in this case?
4. Which of the most common chromosomal abnormality that can be seen in this case?
5. What are the common causes of this condition?

Case 10

A 65-year-old farmer was admitted for evaluation of a progressing anaemia.

Lab findings: Hb 4.8g/dl, TC 7500 cells/µl, DC: N-52%, L-26%, M 7%, E-1%, B-1%, Immature granulocytes (band and meta-myelocytes)-13%, RBC count-1.09 million cells/µl, reticulocyte count-2.0%, reticulocyte index 0.79, PCV-13.2%, MCV-112.9 fl, MCH-34.4 pg, MCHC-30.7 g/dl, RDW-20.3%, platelet- 90,000 cells/ mm^3, iron and vitamin B_{12} levels are within normal limits. PS: Dimorphic anaemia with pancytopenia.

Answer the following questions:

1. What is your diagnosis?
2. What are the morphological abnormalities seen in bone marrow?
3. Name the investigations to be done in this case.

Case 11

A 5-year-old child presented with repeated episodes of fever and upper respiratory infection. On examination: Palllor + , hepato-splenomegaly +.

***Lab investigations*:** Hb-7.2g/dl, TC-22,200 cells/mm^3, DC-lymphocytosis and atypical cells, PCV-29.2%, platelet count-1.30 cells/ mm^3 and ESR-42 mm/hour, alkaline phosphatase 156IU/L, lactate dehydrogenase 1650IU/L and uric acid 5 mg/dl.

***Peripheral smear findings*:** Normocytic normochromic anaemia with large atypical lymphocytes and thrombocytopenia.

Bone marrow aspiration findings: Increased bone marrow cellularity. 92% blast cells with high nuclear cytoplasmic ratio, fine nuclear chromatin and inconspicuous nucleoli. No Auer rods.

Answer the following questions:

1. What is your diagnosis and why?
2. Describe the blasts in this case and what are the cytochemical markers to be used in this case?
3. What are the cytological subtypes in the revised FAB classification of ALL?
4. What are the immunophenotypic markers to be used to confirm the diagnosis?
5. What the prognostic indicators in ALL?

Case 12

A 35 years female with fever, weight loss and bleeding gums.

On examination: Hepatosplenomegaly +, petechiae+.

Investigations done:

- Hb: 2.8 g%.
- TLC: 2,12,000 cells/mm^3.

Peripheral smear—showed plenty of large cells with nucleus occupying 4/5th of the cell and having prominent 3–4 nucleoli.

Answer the following questions:

1. Write the diagnosis?
2. How do you classify this disease (FAB)?
3. What are the other investigations indicated for confirmation.
4. Name some special stains done for confirmation.

Case 13

A 26-month-old male child patient was admitted 9 month ago to the hospital, presenting with neutropenia associated with anaemia and thrombocytopenia. On examination—pallor+, splenic enlargement+. The patient had been suffering from febrile episodes and night sweats, weakness and fatigue. Biochemical parameters—Serum LDH was slightly raised. Peripheral blood smear examination showed normocytic normochromic red blood cells including few nucleated red blood cells, white blood cells showed left shift with significant number of blasts of myeloid series cells.

Bone marrow aspiration and biopsy—showed increased abnormal megakaryocytic, monolobed and multinucleated megakaryocytes, the blasts showed cytoplastic blebbing, the leukemic cells were positive for CD13 , CD33, CD42 and CD61 and negative for CD3, CD5, CD7, CD20, CD22 and human leukocyte antigen-DR.

Answer the following questions:

1. What is the diagnosis of above case?
2. What findings confirm the diagnosis?

Case 14

A 20-year Caucasian male presented with low-grade fever and lethargy. Past medical history was unremarkable. Physical examination showed purpuric lesions on the lower extremities.

There was no lymphadenopathy and hepatosplenomegaly. Further investigations showed Hb 9.5 g/l, WBC count 1.1 lakh and platelets 35 thousand. Peripheral blood showed 80% atypical mononuclear cells.

Coagulation profile—fibrinogen and biochemical profile was unremarkable.

Bone marrow (BM) aspiration showed 80% abnormal promyelocytes with a few promyelocytes showing granulation and Auer rods in many of the cells and bundles.

Answer the following questions:

1. What is the diagnosis of above case?
2. What findings confirm the diagnosis?
3. What is the prognosis of above case?

Case 15

A 74-year-old woman presented with a 2-month history of fatigue and recurrent fever.

***Laboratory investigations*:** TC-16.7 × 10^9/L, RBC count was 1.9 × 10^{12}/L, Hb 6–4g/dl, platelet count 61000 cells/mm^3, PS: WBC increased significantly and granulocytes reduced, monocytes increased significantly (86%). Bone marrow findings: Hypercellular with 80% of the cells to be monoblasts.

Answer the following questions:

1. What is the diagnosis of above case?
2. What findings confirm the diagnosis?

Case 16

A 45-year-old male patient presented with weakness, dyspnoea, pallor, weight loss and massive splenomegaly.

***Laboratory investigations*:** Hb 9.9 g/dl, TC 2,66 × 10^9/L, DC showed basophilia, platelet count increased.

***Bone marrow aspiration*:** Hypercellular marrow with basophilia, eosinophilia, immature granulocytic series and increase in megakaryocytes.

***Peripheral smear*:** Immature granulocytic cells, basophilia and eosinophilia.

Answer the following questions:

1. What is your diagnosis?
2. What is the chromosomal abnormality?
3. What are the phases of the above disease?
4. What are the types of blast crisis and the criteria for diagnosis of blast crisis?

Case 17

A 45-year-old male patient, generalised weakness, weight loss, marked splenomegaly +.

Investigations done:

- Haemoglobin: 9.4 g%.
- TLC 1,02,000 cells/mm^3.

***Peripheral smear*:** Plenty of myeloblasts with immature myeloid series cells and basophils present. Platelets reduced.

Answer the following questions:

1. Write the diagnosis and differential diagnosis?
2. What is the chromosomal abnormality?
3. What is blast crisis?

Case 18

A 55-year-old male patient, generalised lymphadenopathy and mild splenomegaly.

Laboratory investigations: Hb 10 g%, TLC 1,00,000 cells/ mm^3.

***Peripheral smear*:** There is increased number of mature small to medium-sized lymphocytes with clumped chromatin, inconspicuous nucleoli, and scant cytoplasm. Smudge cells seen.

Answer the following questions:

1. Write the diagnosis?
2. What are the complications?
3. What is the bone marrow picture?

Case 19

A 62-year-old female complaints of weight loss, decreased appetite and body pain.

On examination: Left inguinal lymph nodes were enlarged, nontender, hepatosplenomegaly+.

Lab investigations: Hb-12.2g/dL, TLC-59.0 × 10^9/L, DC- N11%, L83%, E1%, M5%, Platelet count-1.8lakh/cumm, LDH–high, Peripheral blood smear-RBCs appear closely packed at places, Rouleaux formation noted. Plenty of mature lymphocytes with dense chromatin and scant cytoplasm were present. Smudge cells were noted.

Answer the following questions:

1. What is your diagnosis and why?
2. What are the further investigations to be done in this case?
3. What are the prognostic factors ?

Case 20

A 16-year-old female presented with petechiae over skin and mucous membrane and menorrhagia since 2 days. A week before she had fever with body ache.

Investigations: Platelet count—58,000 cells/mm^3
Bleeding time—>15 min
Clotting time, PT, aPTT—Normal

***Bone marrow findings*:** Plenty of megakaryocytes, at places in clusters are present. These are immature and hypogranular.

Answer the following questions:

1. What is your diagnosis?
2. What is the pathogenesis of the same? How to differentiate acute from chronic ITP?
3. Mention other condition with thrombocytopenia.

Case 21

A 55-year-old male presented with history of pathological fracture of hip bone. X-ray showed

multiple lytic lesions in iliac bones. ESR 98 mm/1 hour, peripheral smear: Increased Rouleaux formation. Serum creatinine: 2.2 g/dl. Bone marrow: Showed increased plasma cells and plasmablasts >30%.

Answer the following questions:

1. What is the diagnosis?
2. Name the urinary finding in the above condition.
3. What are the radiological findings?
4. What are the complications?

Case 22

A 68-year-old hypertensive male came was admitted for pain abdomen, oliguria and easy fatigability.

***Laboratory investigation*:** Hb 9.2 g/dl, TLC 3,482 cells/mm^3, platelet count 2.8 lakh/mm^3, ESR 108 mm/hour. Serum creatinine 4.8 mg/dl, S. calcium 2.4 mg/dl, albumin 2.3 g/dl, globulin 6.4 g/dl (Reversal of albumin globulin ratio) and C-reactive protein 20 mg/L. X-ray—lytic lesion over tibia and skull.

PS: Normocytic normochromic anaemia with increased Rouleux formation.

Answer the following questions:

1. What is your diagnosis?
2. What are its variants?
3. What are the other investigations to be done in this case?
4. What is Bence–Jones protein?